# INTEGRIN AFFINITY MODULATION BY RAS SIGNALLING MOLECULES

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# DECLARATION

I hereby declare that this thesis has been composed solely by myself and has not been accepted in any previous candidature for a higher degree. All work presented in this thesis, was, unless acknowledged, initiated and executed by myself. All sources of information in the text have been acknowledged by reference.

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# ABSTRACT

Integrins are heterodimeric transmembrane proteins present on virtually every cell, which maintain and control cell-cell and cell-substratum adhesion. Through this adhesive property, integrins can govern aggregation, cell polarity and migration. Several human diseases demonstrate the importance of integrins in normal physiological function: inappropriate platelet integrin activation is responsible for myocardial infarctions and cerebral vascular diseases; while leucocytes require integrin activation for leucocyte migration during inflammation. The understanding of the function and regulation of integrins therefore has important therapeutic implications.

Essential to the function of integrins is their ability to modulate the affinity for binding ligand through conformational change. Platelet aggregation requires prior activation of the $\alpha_{IIb}\beta_3$ integrin to bind fibrinogen. This platelet integrin is a model for the understanding of the mechanisms regulating affinity modulation. A high affinity state-specific antibody, PAC1 has facilitated the analysis of $\alpha_{IIb}\beta_3$ activation. Intracellular signal transduction pathways elucidated through the use of the PAC1 antibody have shown that the integrin cytoplasmic domains are required for affinity modulation, in a process termed "inside-out signalling".

In this thesis I have sought to understand the mechanism by which H-Ras and its effectors modulate integrin affinity. H-Ras is a member of the Ras superfamily of small GTP binding proteins. Expression of the constitutively active variant of H-Ras (Ras G12V) within an integrin affinity reporter system ($\alpha\beta$-py cells) reduced integrin affinity (suppressed integrin). Ras effector mutants revealed that integrin suppression is mediated by Raf-dependent and Raf-independent signalling pathways. Raf-independent signalling pathways activated by Ral-GEFs and PI3-kinase were not responsible for integrin suppression. An active variant of R-Ras (R-Ras G38V) reversed integrin suppression by both Raf-dependent and –independent pathways, indicating that these pathways may converge at a point proximal to the integrin.

Raf initiates a protein signalling cascade leading to ERK activation that is responsible for many of the Ras/Raf-dependent biological functions. However, Raf-dependent integrin suppression was insensitive to MEK inhibition with the PD098059 compound. A novel Raf mutant (T481A) that fails to bind to MEK was also capable of mediating integrin suppression in the absence of ERK activation. Surprisingly, Raf-BxB T481A-mediated integrin suppression was sensitive to expression of MKP-1. Taken together it is proposed that Raf may mediate integrin suppression via a MEK-independent pathway that may utilise a member of the MAP kinase superfamily.

In conclusion, integrin suppression by Ras is mediated by both Raf-independent and dependent pathways. Signalling by Raf may utilise components other than those present in the classical Ras to ERK protein cascade.

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# ABBREVIATIONS

| | |
|---|---|
| AI | Activation Index |
| Ala | Alanine |
| APS | Ammonium persulphate |
| ATP | Adenosine 5' triphosphate |
| AU | Arbitrary Units |
| Bcl-2 | B cell leukaemia oncogene 2 |
| bp | Base pair |
| BSA | Bovine serum albumin |
| $Ca^{2+}$ | Calcium |
| CAAX | Cysteine-Aliphatic amino acid-Aliphatic amino acid-Serine/Methionine |
| $CaCl_2$ | Calcium chloride |
| CD | Cluster of differentiation |
| cDNA | Complimentary DNA |
| CHO | Chinese hamster ovary |
| CMV | Cytomegalovirus |
| DAG | Diacylglycerol |
| DMEM | Dulbeccos Modified Eagles Medium |
| DMSO | Dimethylsulphoxide |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynuclotide triphosphate |
| DTT | Dithiotreitol |
| E.Coli | Escherichia Coli |
| E37G | Glutamic $Acid^{37} \rightarrow Glycine^{37}$ |
| ECL | Enhanced chemiluminescence |
| ECM | Extracellular Matrix |
| EDTA | Ethylenediaminetetraacetic acid |
| EGF | Epidermal growth factor |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| EGTA | Ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid |
| ERK | Extracellular signal-regulated kinase |
| FACS | Fluorescence activated cell sorter |
| FAK | Focal adhesion kinase |
| FBS | Foetal bovine serum |
| Fg | Fibrinogen |
| FITC | Fluorescein isothiocyanate |
| FN | Fibronectin |
| G12V | $Glycine^{12} \rightarrow Valine^{12}$ |
| G418 | Geneticin |
| GAP | GTPase Activating Protein |
| GDP | Guanine diphosphate |
| GEF | Guanine nucleotide Exchange Factor |
| Gly | Glycine |
| Grb2 | Growth-factor-receptor-bound protein 2 |
| GST | Glutathione-s transferase |
| GTP | Guanine triphosphate |
| HCl | Hydrochloric acid |
| HEPES | EGTAEthylene glycol-bis (β-aminoethyl ether) N,N,N'N'-tetracetic acid |
| HRP | Horse radish peroxidase |
| IC50 | Inhibitory concentration 50% |

| | |
|---|---|
| ICAM | Immunoglobulin-like Cell Adhesion Molecule |
| $IP_3$ | Inosito 1,4,5-trisphosphate |
| IPTG | Isopropylthio-β-D-galctoside |
| JNK | c-Jun N-terminal kinase |
| kb | Kilobase |
| kDa | Kilodalton |
| LB Media | Luria-Bertani Media |
| LN | Laminin |
| M | Molar |
| MAPK | Mitogen activated protein kinase |
| MBP | Myelin Basic Protein |
| MEK | MAPK/ERK Kinase |
| $MgCl_2$ | Magnesium chloride |
| MKP | MAP Kinase Phosphatase |
| $Mn^{2+}$ | $Managenese^{2+}$ |
| mRNA | Messenger ribonucleic acid. |
| NaCl | Sodium chloride |
| NaF | Sodium flouride |
| OD | Optical density |
| $p70^{s6k}$ | Ribosomal protein S6 kinase |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PD | PD098059 |
| PDGF | Platelet derived growth factor |
| pERK | Phosphorylated ERK |
| PI3-kinase | Phosphoinositide-3-OH kinase |
| PKC | Protein kinase C |
| PLA2 | Phospholipase A2 |
| PLC | Phospholipase C |
| PLD | Phospholipase D |
| PS | Phosphatidylserine |
| PtdIns/PI | Phosphatidylinositol |
| RalBD | Ral Binding Domain of RLIP76 |
| RalGDS | Ral guanine nucleotide dissociation stimulator |
| RalGEF | Ral guanine nucleotide exchange factor |
| RGD | Arginine-Glycine-Aspartic Acid |
| Rgl | RalGDS-like Factor |
| Rlf | RalGDS-like |
| R-PE | R-phycoerythrin |
| RTK | Receptor tyrosine kinases |
| SCLC | Small cell lung cancer |
| SDS | Sodium dodecylsulphate |
| SDS-PAGE | Sodium-dodecyl-sulfate polyacrylamide gel electro-phoresis |
| SEM | Standard error of the mean |
| SH | Src homology |
| Shc | SH2-domain-containing α2-collagen related protein |
| SOS | Son of Sevenless |
| Src | Rous sarcoma virus |
| T35S | $Threonine^{35} \rightarrow Serine^{35}$ |
| Thr | Threonine |
| TM | Transmembrane region |
| Trp | Tryptophan |

| | |
|---|---|
| VCAM | Vascular Cell Adhesion Molecule |
| VEGF | Vaculae Endothelial Growth Factor |
| Vn | Vitronectin |
| WT | Wildtype |
| Y40C | $Tyrosine^{40} \rightarrow Cysteine^{40}$ |

# INTRODUCTION: CHAPTER 1

# Integrins and Integrin Affinity

Integrins are cell surface receptors that bind to extracellular matrix (ECM) proteins. Much of the initial information regarding integrins was largely a result of studies performed on three main groups of proteins: the fibronectin receptor on chick fibroblasts and osteosarcoma cells, platelet glycoprotein GPIIb-IIIa and the leucocyte antigens (LFA-1, Mac-1 and the VLA antigens). These proteins were shown to be composed of two non-covalently attached subunits, termed α and β. Peptide sequencing and cloning of these receptor subunits revealed sequence homology between the α and β subunits from these different protein groups. The term "integrin" was used as a generic term to describe this family of adhesion receptors and the αβ nomenclature adopted to simplify and classify the subunits according to their sequence identity (Hynes, 1987; Ruoslahti and Pierschbacher, 1987).

## 1.1 Multiple integrin subunits and ligands

All integrins are composed of an alpha and beta subunit non-covalently attached on the cell surface. Since the initial reviews relating to integrins in which three β subunits were described, the number has increased to 18α and 8β subunits. Sequencing of the human genome suggests that there may be as many as 24α and 9β subunits. Mathematically, these subunits could associate to produce more than 100 different αβ heterodimers. The *in vivo* pattern of αβ heterodimerisation appears to be much more restricted, with only 24 different receptors identified (Danen and Yamada, 2001; Plow *et al.*, 2000). Several α subunits, such as $\alpha_{IIb}$, associate exclusively to a single β subunit; in contrast other α subunits such as $\alpha_V$ are capable of associating with several β subunits. These αβ heterodimers allow the integrins to be classified into subgroups according to their β subunit interactions (Table 1.1). The complexity of αβ heterodimers is further expanded by alternative splicing of

integrin subunits. Splice variants have been described for both α and β subunits, with changes in both the extracellular and the intracellular domains (de Melker and Sonnenberg, 1999).

Many of the integrins were described prior to the αβ nomenclature classification of integrins. The platelet integrin $\alpha_{IIb}\beta_3$ is often referred to as GPIIb-IIIa and several $\beta_1$ integrins have either a platelet glycoprotein (GP) nomenclature or are known as VLA (very late after activation) antigens. Only the $\beta_2$ integrins are routinely referred to in the literature by their earlier names: $\alpha_L\beta_2$ – lymphocyte function associated antigen-1 (LFA-1) and $\alpha_M\beta_2$ – macrophage-1 receptor (Mac-1) (see Table 1.1).

Integrins are expressed on virtually every mammalian cell and expression profiles of integrins are just as complex as their heterodimer interactions. The $\beta_1$ integrins are the most broadly expressed, whereas $\beta_2$ integrin expression is restricted to leucocytes (Springer, 1990). Of the $\beta_3$ integrins; $\alpha_{IIb}\beta_3$ is primarily found on platelets, but expression has also been observed in cells of the megakaryocyte lineage and some tumours; while $\alpha_V\beta_3$ has a much broader expression profile including platelets, endothelial cells and monocytes (Smyth *et al.*, 1993).

### 1.1.1 Evolution of integrins

Integrin subunits have been identified in several organisms ranging from sponges, corals, nematodes and echinoderms to mammals (Hynes and Zhao, 2000). The process of integrin-mediated adhesion therefore appears to be evolutionarily conserved. Studies performed in *C. elegans*, *Drosophila* and vertebrates show that each has a basic set of ECM-binding integrins, one laminin-specific and one recognising an Arg-Gly-Asp (RGD) motif, that have been preserved throughout evolution. Expansion from this basic set of integrins and evolution of new integrins has occurred in vertebrates giving rise to the large integrin number within the human genome (Brown, 2000).

**Table 1.1 Integrin heterodimers and their ligands.**

| Subunits | Previous Names | Fn | Lam | Coll | Fg | Vn | Other |
|---|---|---|---|---|---|---|---|
| $\beta_1$ Integrins<br>$\alpha_1\beta_1$ | VLA-1, CD41a/CD29 | | X | X | | | |
| $\alpha_2\beta_1$ | VLA-2, CD41b/CD29 | X | X | X | | | Thr |
| $\alpha_3\beta_1$ | VLA-3, CD41c/CD29 | X | X | X | | | VCAM-1 |
| $\alpha_4\beta_1$ | VLA-4, CD41d/CD29 | X | | | | | |
| $\alpha_5\beta_1$ | VLA-5, CD41e/CD29 | X | X | | X | | |
| $\alpha_6\beta_1$ | VLA-6, CD41f/CD29 | | X | | | | |
| $\alpha_7\beta_1$ | | | X | | | | |
| $\alpha_8\beta_1$ | | X | | | | | Tenascin |
| $\alpha_9\beta_1$ | | | | | | | Tenascin |
| $\alpha_{10}\beta_1$ | | | | X | | | |
| $\alpha_{11}\beta_1$ | | | | X | | | |
| $\alpha_V\beta_1$ | CD51/CD29 | X | | | | X | |
| $\beta_2$ Integrins<br>$\alpha_L\beta_2$ | LFA-1, CD11a/CD18 | | | | | | ICAM 1-5 |
| $\alpha_M\beta_2$ | Mac-1, CD11b/CD18 | | | | X | | ICAM-1,<br>Fx, C3bi |
| $\alpha_X\beta_2$ | P150/95, CD11c/CD18 | | | | X | | C3bi |
| $\alpha_D\beta_2$ | | | | | | | VCAM-1 |
| $\beta_3$ Integrins<br>$\alpha_{IIb}\beta_3$ | GPIIIb/IIa, CD41/CD61 | X | | X | X | X | Disintegrins,<br>vWF, Thr |
| $\alpha_V\beta_3$ | CD51/CD61 | X | X | | X | X | As $\alpha_{IIb}\beta_3$ and tenascin,<br>osteopontin and Bs |
| Other Integrins<br>$\alpha_6\beta_4$ | | | X | | | | BM |
| $\alpha_V\beta_5$ | | X | | | | X | Bs |
| $\alpha_V\beta_6$ | | X | | | | | Tenascin |
| $\alpha_4\beta_7$ | | X | | | | | VCAM-1,<br>MADCAM-1 |
| $\alpha_E\beta_7$ | | | | | | | E-Cadherin |
| $\alpha_V\beta_8$ | | X | | | | | |

X denotes an interaction. *Abbreviations:* Fn - fibronectin; Lam - laminin; Coll - collagens; Fg - fibrinogen; Vm - vitronectin; Thr - thrombospondin; Fx - factor X; vWF - von Willebrand factor; BM - basement membrane and Bs - bone sialoprotein (de Melker and Sonnenberg, 1999; Hynes, 1992; Plow *et al.*, 2000).

### 1.1.2 Integrin ligands

Integrins were initially described by their ability to bind to extracellular matrix proteins (fibronectin, laminin, collagen and vitronectin) and mediate cell-substratum adhesion. Other ligands such as fibrinogen, a soluble plasma protein and integral membrane proteins (ICAM-1/2 and VCAM-1) mediate cell-cell adhesion.

Similar to the αβ heterodimer interactions, individual integrins can bind multiple ligands and individual ligands are recognised by several integrins. Put simply this allows a cell to bind to numerous ligands on the basement membrane and on neighbouring cells. The interactions between integrins and ligands are summarised in Table 1.1.

Fibronectin (Fn) is a heterodimeric glycoprotein of approximately 250kDa. Alternative splicing of the fibronectin gene in humans can generate 20 different isoforms that dimerise to form the final product. Fibronectin is an extended and flexible molecule composed of multiple globular domains of three repeat sequences known as type I, II and III FN repeats. Plasma and cellular fibronectin are both substrates for fibronectin fibril and matrix formation in an integrin-dependent cellular process. Fibronectin has extensively been used as a prototype integrin ligand in the study of integrin-mediated adhesion and integrin signalling (Romberger, 1997)

Laminin is a heterotrimeric glycoprotein present in the basement membrane. The three laminin subunits form a cruciform structure that is held together with disulphide bonds. Isoforms of the laminin subunits allow the formation of different laminin molecules that display distinct tissue expression patterns. Within the basement membrane, laminin can bind to other ECM proteins including collagen IV, heparan sulphate proteoglycans, entactin and to itself. Laminin has a variety of biological activities including cell adhesion, growth, migration, neurite outgrowth and tumour metastasis (Malinda and Kleinman, 1996).

Fibrinogen is a soluble plasma protein of 340kDa. Synthesised primarily by hepatocytes, this heterotrimer forms a symmetrical protein held together by 29

disulphide bonds. Fibrinogen can lead to cross-linking of integrin receptors ($\alpha_{IIb}\beta_3$) essential during the formation of a fibrin clot (Herrick *et al.*, 1999).

Integral membrane proteins also serve as integrin ligands mediating cell-cell adhesion. These ligands are part of the immunoglobulin superfamily and include the ICAMs (Ig-like cell adhesion molecule) and VCAM-1 (vascular cell adhesion molecule). The extracellular domain is composed of immunoglobulin-like repeat units that vary in number between the different ligands (Petruzzelli *et al.*, 1999).

Within these large ligand macromolecules, specific recognition sequences have been identified that are crucial for ligand binding. The Arg-Gly-Asp (RGD) sequence present in fibronectin is essential for binding to the fibronectin receptor $\alpha_5\beta_1$ and soluble RGD peptides can inhibit fibronectin-integrin binding. The presence of RGD sequences in multiple ligands suggests that ligand specificity may require other recognition sequences. Recognition sites other than RGD have been identified in both fibronectin and fibrinogen (Plow *et al.*, 2000).

## 1.2 Integrin structure

Integrins are a heterodimer composed of an $\alpha$ (150-200kDa) and $\beta$ (90-110kDa) subunit. Each contains a large N-terminal extracellular domain, a single transmembrane region and a short (<50 amino acids, except $\beta_4$, >1000) cytoplasmic region. The integrin subunits interact to form the integrin heterodimer; sequence and structure predictions reveal several domains within the $\alpha$ and $\beta$ subunits (Hynes, 1992).

Electron microscopy of the $\alpha_{IIb}\beta_3$ integrin revealed a molecule with a globular oblong head and two rod-like tails (Carrell *et al.*, 1985). Most of the proposed integrin structures have relied on primary sequence analysis and structure predictions using homology to proteins to which tertiary structure has been solved: Figure 1.1 shows a schematic representation of the domain structure of an integrin dimer.

The N-terminal region of all $\alpha$ subunits contains a seven-fold repeat region, encoding 4 $\beta$-sheets that come together in a structure known as a $\beta$-propeller (Springer, 1997).

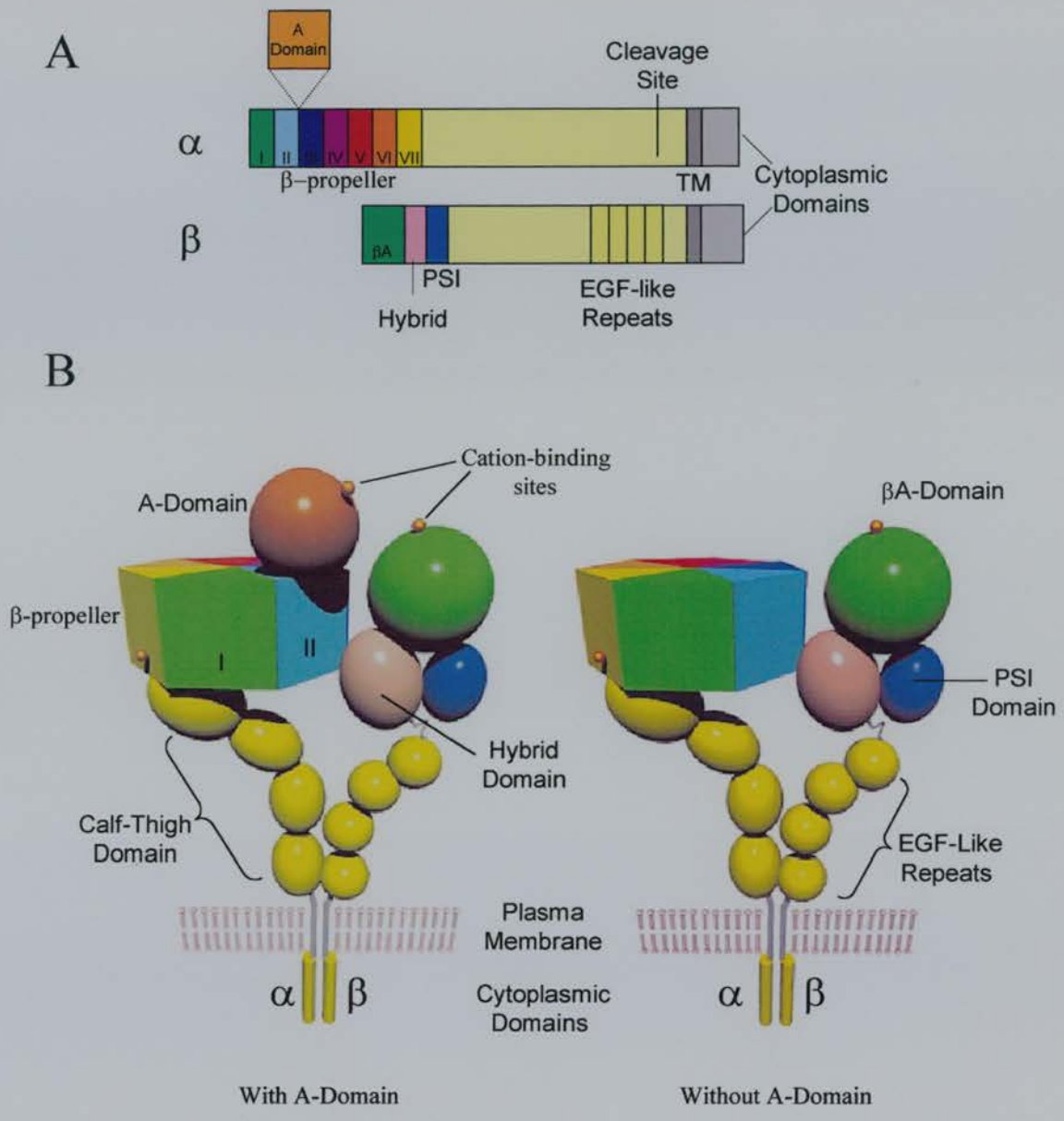


**Figure 1.1 Schematic of integrin structure.**

(A) Linear diagram of the domain structure of the α and β subunits. The cleavage site indicates where the post-translational cleavage may occur with some α subunits. The αA-domain is inserted between repeat II and III of the β-propeller structure. (B) Modular structure of a 3-D representation of an integrin with and without an αA-domain (modified from Humphries 2000).

Within repeats number 4-7 there are sequences (EF-hand homology) that display divalent cation binding properties.

Alpha subunits $\alpha_1$, $\alpha_2$, $\alpha_L$, $\alpha_M$ and $\alpha_X$ posses an additional A-domain between repeat 2 and 3, which contains a dinucleotide binding fold of β-strands and surrounding α-helices (Lee *et al.*, 1995a). Adjacent to the β-propeller domain are several modules made up of β-sheets, known as the thigh and calf-domain. Some α subunits are post-translationally cleaved prior to the transmembrane region to yield an N-terminal heavy and C-terminal light chain.

The β subunits also contain an A-domain-like region that contains several metal ion-dependent adhesion sites (MIDAS). Connecting the A-domain and the EGF-like region is the hybrid domain and a module called the PSI region. Four cysteine-rich EGF-like regions link the extracellular region to the transmembrane sequences (Humphries, 2000).

The ligand binding pocket has been mapped to the interface of the α subunit β-propeller domain and β subunit A-domain (Figure 1.2A) (Humphries, 2000). In integrins containing an αA-domain, the major ligand binding sites are located within the αA-domain rather than the β-propeller region. The crystal structure of the extracellular segment of $\alpha_V\beta_3$ revealed that the αβ interface was very similar to that of the Gα-Gβ interface of G-proteins (Xiong *et al.*, 2001). G-proteins undergo major conformational changes upon GTP hydrolysis; so possibly changes at the αβ interface may explain conformational changes within the integrin during ligand-binding/activation. The presence of a metal-ion binding site in the putative ligand binding region may explain observations relating to the regulatory effects of metal ions on ligand binding (Figure 1.2B).

The short cytoplasmic domains link the integrins with the cytoskeleton, either directly or via adapter proteins (talin and α-actinin). The subunits also contain several phosphorylation sites for protein kinases. The large cytoplasmic domain of $\beta_4$ localises this integrin to hemidesmosomes in epithelial cells where it interacts with intermediate filaments.

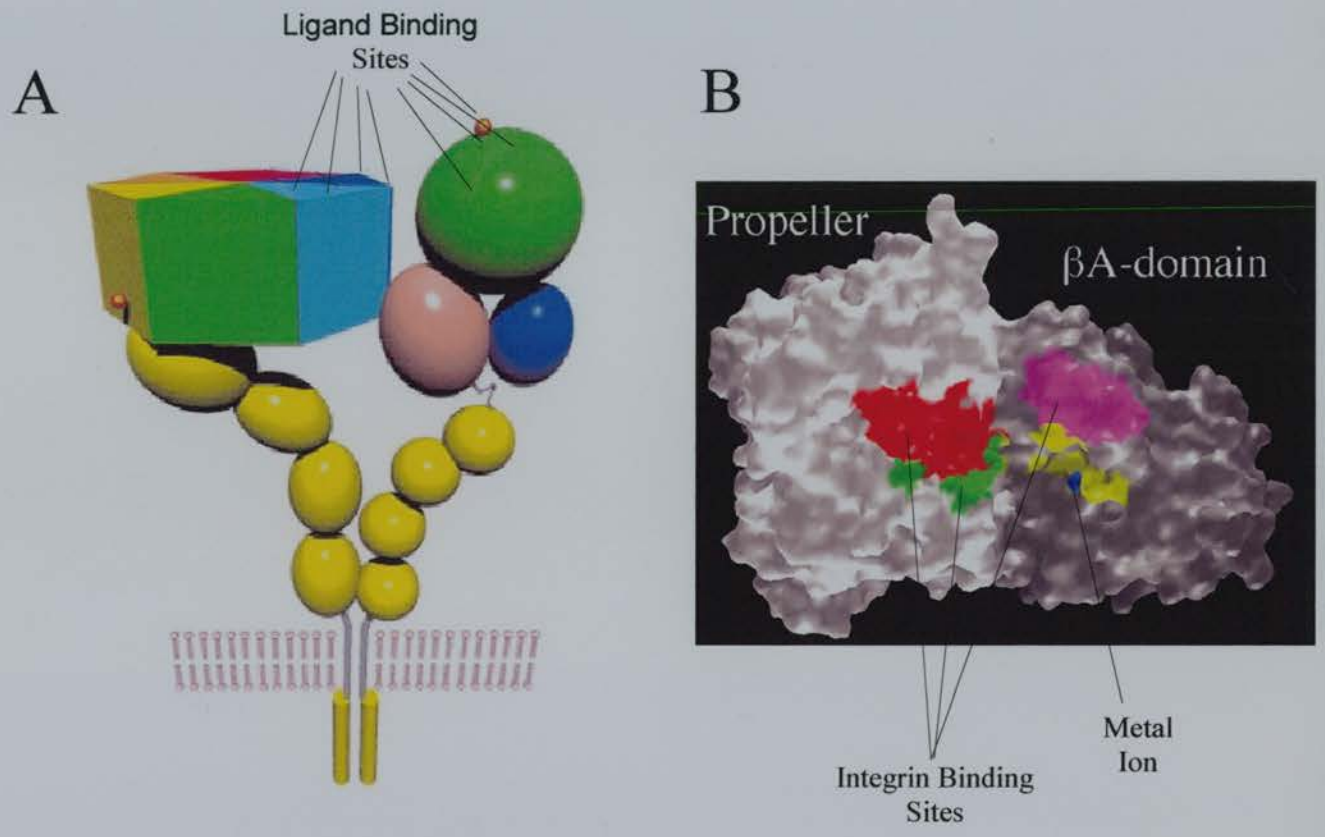


**Figure 1.2 Integrin ligand binding sites.**

(A) Modular representation of the ligand binding sites in a 3-D integrin model (Humphries 2000). (B) Ligand binding sites within an $\alpha_v\beta_3$ representation viewed from the top of the head (tails lying in the back). At the αβ interface, ligand binding sites (red, green and magenta) are present on both subunits. Residues important for the MIDAS site are shown in yellow (Xiong *et al.*, 2001).

## 1.3 Cellular role of Integrins

Integrins were identified as receptors for ECM proteins. The link between ECM proteins and the cytoskeleton by integrins allows cell adhesion to the basement membrane. Adhesion to the ECM occurs at specialised sites within the plasma membrane called focal adhesions or focal contacts. Focal adhesions are large adhesion contacts located at the ends of actin stress fibres, whereas focal contacts are much smaller and located at the tips of extending filopodia and lamellipodia (Petit and Thiery, 2000). These sites are characterised by clusters of ligand-occupied integrins linked to actin bundles by large multimeric protein complexes.

These cell-matrix adhesions are far from simple contact sites between the cell and the ECM - they play essential biological roles during cell migration, cell growth and cell survival. The $\beta_1$ tail has been shown to be essential for integrin localisation to focal adhesions (Hayashi *et al.*, 1990; Solowska *et al.*, 1989). In contrast, $\alpha$ tails may regulate the ability of $\alpha\beta$ heterodimers to localise to these sites (Briesewitz *et al.*, 1993; Ylanne *et al.*, 1993). Considerable effort has been directed at the understanding of focal adhesion formation and their role in biological processes.

Focal adhesion formation is initiated by ligand-induced integrin clustering and is summarised in Figure 1.3 (Miyamoto *et al.*, 1995a; Miyamoto *et al.*, 1995b; Petit and Thiery, 2000). Conformational changes have been observed in the cytoplasmic domain of $\alpha_{IIb}\beta_3$ upon ligand binding, suggesting that following ligand binding new protein interactions may occur (Leisner *et al.*, 1999). Integrin clustering requires Rho activity (Hotchin and Hall, 1995; Ridley and Hall, 1992), which may be mediated by integrin-ligand binding (Ren *et al.*, 1999). Following integrin clustering, actin-binding proteins (talin, filamin and $\alpha$-actinin) (Critchley, 2000; Miyamoto *et al.*, 1995a; Miyamoto *et al.*, 1995b) and focal adhesion kinase (FAK) complex with integrin cytoplasmic tails (Hanks *et al.*, 1992; Schaller *et al.*, 1992). Ligand-induced activation of FAK at the forming focal adhesion leads to autophosphosphorylation and the formation of a SH2 binding domain (Schaller *et al.*, 1992; Schaller *et al.*, 1994). Recruitment of Src kinase to the focal adhesion and subsequent tyrosine

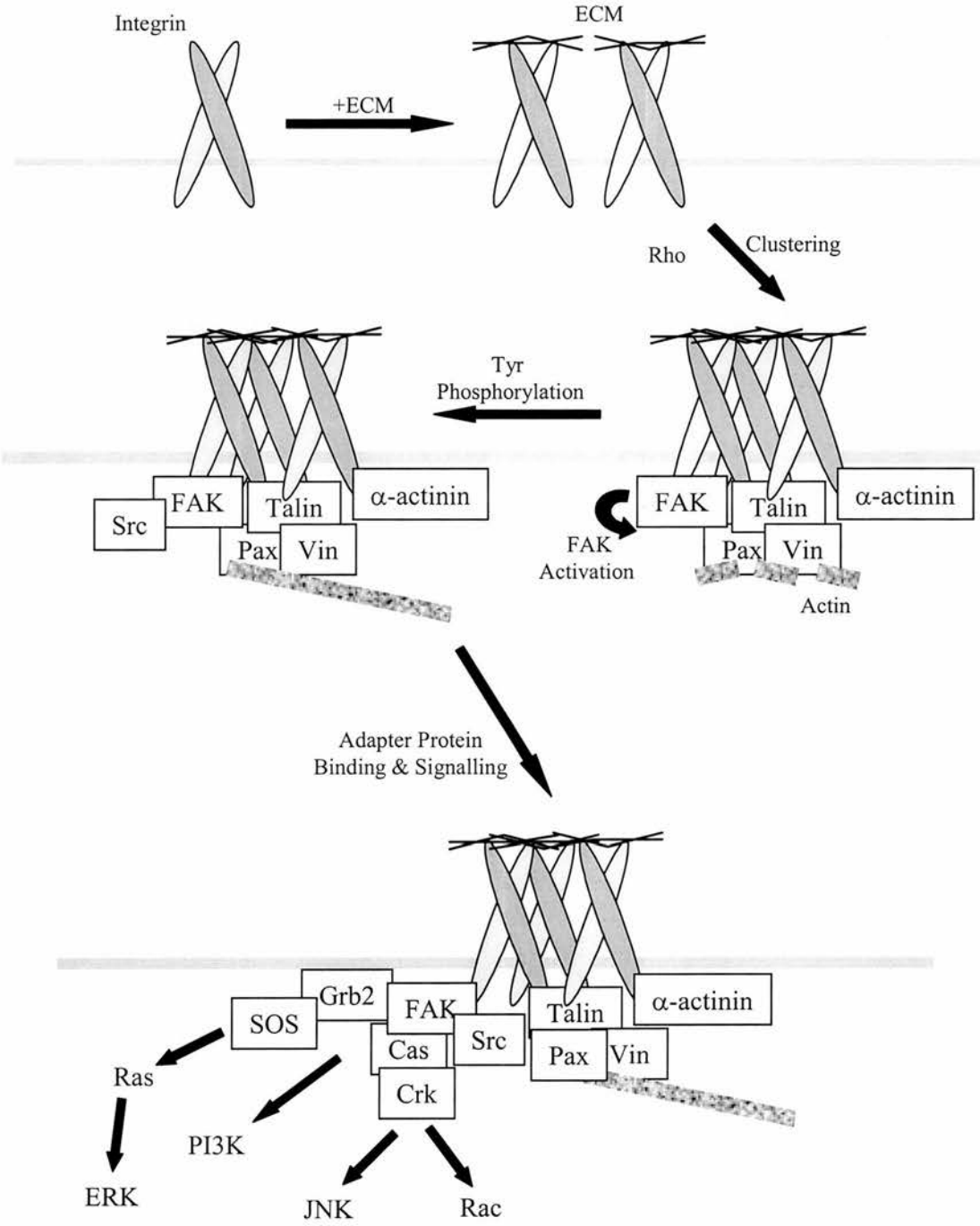


**Figure 1.3 Focal adhesion formation.**

Matrix binding to integrins promotes clustering and binding of cytoskeletal adaptor proteins, FAK and actin. FAK activation allows Src binding; subsequent tyrosine phosphorylation generates binding sites for several adaptor proteins leading to activation of multiple signalling pathways.

phosphorylation of FAK leads to the formation of SH2 and SH3 binding sites for several signalling adapter/docking proteins (Giancotti and Ruoslahti, 1999). Recruitment of p130cas/crk (Vuori *et al.*, 1996) and Grb2/SOS (Schlaepfer *et al.*, 1994) results in the activation of Rac/JNK and Ras signalling pathways respectively. Grb2/SOS is also recruited to focal adhesions by the adapter protein Shc via Fyn kinase (Wary *et al.*, 1996). Active FAK may also activate the phosphoinositide 3-OH kinase (PI3-kinase) pathway (Chen *et al.*, 1996). Signalling pathways activated in response to integrin-ligand binding (outside-in signalling) are essential for the effects on cell cycle control and cell survival (see below).

The link between the actin cytoskeleton and the extracellular matrix by integrins generates tension within the cell (Horwitz and Parsons, 1999). The adhesive strength has been mathematically related to cell migration rates. The rate of migration may be plotted as a bell-shaped curve against the strength of cell adhesion: lack of sufficient traction, with poor cell adhesion, results in low migration rates; migration reaches an optimum as cell adhesion increases, due to increases in cell traction; beyond this point, strong cell adhesions fail to retract, thereby reducing migration rates again (Holly *et al.*, 2000; Palecek *et al.*, 1997).

### 1.3.1 Control of cell cycle by integrins

The multiple biological effects attributed to integrin-ligand binding are a consequence of the signalling molecules recruited to focal adhesions. Signals emanating from focal adhesions can co-operate with growth factor receptor signalling pathways for cell cycle progression. Optimal growth factor signalling in many cells is only observed in adherent cells. The $\alpha_V\beta_3$ integrin can associate with the PDGF, insulin and VEGF receptors while some $\beta_1$ integrins interact with the EGF receptor (Giancotti and Ruoslahti, 1999). Such complexes of integrins and receptors may be required for optimal mitogenic signal pathway activation (Kumar, 1998; Miyamoto *et al.*, 1996). Direct activation of Ras and PI3-kinase pathways at the focal adhesions also contributes to cell cycle progression. Upregulation of cyclin-D1 (Fang *et al.*, 1996) and down-regulation of Cdk2 inhibitors (Zhu *et al.*, 1996) are

associated with anchorage-dependence in progression through the G1 phase of the cell cycle.

### 1.3.2 Role of integrins in cell survival

Epithelial and endothelial cells that detach from the extracellular matrix undergo apoptosis in a process termed anoikis (Frisch and Francis, 1994; Meredith, Jr. *et al.*, 1993). This is a mechanism to ensure that cells displaced from their natural environment are removed before they colonise another area. Similar to cell cycle control, anoikis displays integrin specificity. Anoikis of CHO cells was inhibited in cells expressing $\alpha_5\beta_1$ but not $\alpha_V\beta_1$ on a fibronectin matrix (Zhang *et al.*, 1995). Cell signalling from focal adhesions appears to provide the anchorage-dependent cell survival signals. Activation of the PI3-kinase/Akt pathway by either FAK or Ras appears to be important in the cell survival signal (Khwaja *et al.*, 1997) and may mediate the anchorage-independent survival. CHO cells expressing $\alpha_5\beta_1$ display an increase in bcl-2 expression on a fibronectin matrix, while $\alpha_V\beta_1$ cells did not and the cells underwent apoptosis. Thus modulation of bcl-2 expression may provide an alternative cell survival signal (Zhang *et al.*, 1995). The balance between cell cycle progression and apoptosis is reflected in a balance between ERK and JNK activity, with loss of ERK activity in detached cells pushing the balance towards apoptosis (Xia *et al.*, 1995). Pathways regulating cell cycle progression and cell survival by integrin-mediated adhesion are summarised in Figure 1.4.

### 1.3.3 Importance of integrins and ligands *in vivo*

*In vitro* experiments have revealed that integrins play an important role in many cellular processes. Many groups have studied the *in vivo* role of integrins and their ligands, in particular with knockout animals. Most of the ECM proteins and integrin subunits have been mutated by homologous recombination (reviewed in Hynes 1996). Ablation of the fibronectin gene produces early embryonic lethality, suggesting that fibronectin is essential during development (George *et al.*, 1993). In contrast, knockouts of fibrinogen, thrombospondin and vitronectin produced viable

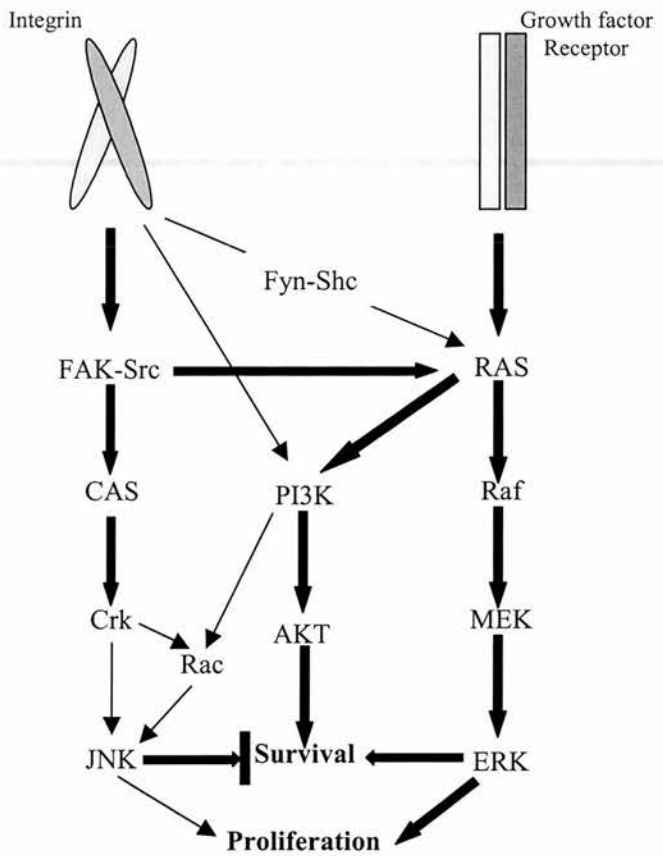


**Figure 1.4 Integrin and growth factor receptor signalling.**

Signalling pathways activated by integrins and growth factors are shown to control cell cycle progression and mediate cell survival. Signalling events that are well characterised are shown by bold arrows (Giancotti and Ruoslahti, 1999; Kumar, 1998).

animals with no obvious developmental defects (Hynes, 1996). The failure to detect developmental defects may reflect that gene specific systems have yet to be identified or that they may play more important roles in the adult animal.

Loss of the $\beta_1$ subunit causes embryonic lethality; this is not surprising as expression of more than 10 different integrin heterodimers is lost (Fassler and Meyer, 1995; Stephens *et al.*, 1995). Losses of individual $\beta_1$-associating $\alpha$ subunits are mostly lethal except for the $\alpha_1$ subunit. Mutations of the other integrin subunits reveal that each have specific *in vivo* roles. This is demonstrated by the different fibronectin integrin knockouts: each produces different phenotypes and none are severe as the fibronectin-null phenotype. These experiments suggest that the different fibronectin integrins each have independent non-overlapping functions during development (Hynes, 1996).

## 1.4 Integrins and disease

Knockout studies have revealed that integrins play important roles during development. Human diseases show that loss of proper control of integrin expression or signalling can have profound effects on disease pathology. Rare genetic disorders affecting integrin expression have been described in humans.

Leucocyte adhesion defiency-1 (LAD-1) results from a loss of $\beta_2$ expression. Germline mutations within the $\beta_2$ gene disrupt $\alpha\beta$ heterodimer formation and result in a loss of LFA-1, Mac-1, p150, p95 and $\alpha_D\beta_2$ integrin expression (Anderson and Springer, 1987). The disease is characterised by recurrent soft tissue infections, primarily a result of the absence of neutrophil infiltration at the site of tissue injury, since leucocyte infiltration requires $\beta_2$ integrin-mediated adherence to endothelia (see below). Glanzmann thrombasthenia results from the loss of the $\alpha_{IIb}\beta_3$ integrin on platelets. The absence of $\alpha_{IIb}\beta_3$-mediated fibrinogen crosslinking, essential for clot formation, results in excessive bleeding of gums, cuts and wounds (Hogg and Bates, 2000). Mutations have been observed both in the $\alpha_{IIb}$ and the $\beta_3$ subunits (Newman *et al.*, 1991). Mutations in which $\alpha_{IIb}\beta_3$ fails to crosslink fibrinogen, with normal

integrin expression, have also been described and are associated with integrin activation (see below). These two disorders highlight two important processes observed with human disease, cell recruitment and platelet aggregation.

An increase in monocyte and T-lymphocyte recruitment is observed in the formation of atherosclerotic plaques in a $\beta_2$-dependent process. The release of several cytokines increases recruitment of smooth muscle cells and further monocyte infiltrates into the plaque. Increases in matrix deposition, foam cell formation and cellular infiltration leads to expansion of the plaque. Rupture of the plaque or endothelial damage causes platelet aggregation to protect the exposed endothelium. Aggregation occurs in an $\alpha_{IIb}\beta_3$-dependent process, leading to thrombus formation. Excessive aggregation can cause further complications with embolism and blockage of small blood vessels (Clemetson and Clemetson, 1998; Hillis and Flapan, 1998).

Asthma is characterised by excessive eosinophil recruitment and degranulation within the lung (Bousquet *et al.*, 1990; Wardlaw, 1999). Eosinophil recruitment is facilitated by increases in both $\alpha_4\beta_1$ and ICAM-1 on the bronchial epithelium and $\beta_2$ integrins on eosinophils (Hillis and MacLeod, 1996).

Excessive matrix deposition is observed in lung granulomas; this increased fibronectin matrix assembly is associated with an increase in $\alpha_2\beta_1$ and $\alpha_5\beta_1$ expression (Roman *et al.*, 1995).

Anchorage independence is a hallmark of tumour cell growth. Mutations in several genes associated with integrin signalling have been observed in tumour cells allowing anchorage independence. Changes in the integrin expression profile have also been observed in many tumours (Varner and Cheresh, 1996). Expression of $\alpha_5\beta_1$ is reduced in transformed cells (Plantefaber and Hynes, 1989). Overexpression of $\alpha_5\beta_1$ in CHO cells increased fibronectin matrix assembly and inhibited tumour growth when injected into nude mice (Giancotti and Ruoslahti, 1990). Loss of $\alpha_2\beta_1$ correlates with the transformed phenotype in breast epithelial cells and ectopic expression of $\alpha_2\beta_1$ in these cells can suppress growth (Zutter *et al.*, 1995). An increase in $\alpha_V$ expression is associated with metastatic melanoma cells and an

inhibitory $\alpha_V$ antibody prevented melanoma tumour growth in nude mice (Felding-Habermann *et al.*, 1992; Mitjans *et al.*, 1995). Changes in the integrin profile also aid tumour cell invasion and upregulation of $\alpha_V\beta_3$ is required during tumour angiogenesis (reviewed in Varner 1996). These specific changes in the cell-integrin profile facilitate tumour cell transformation and metastasis.

## 1.5 Modulation of ligand binding strength

Ligand-integrin specificity is determined by interactions within the ligand binding site of the integrin. The ability of an integrin to bind to its ligand also depends on the activity of the integrin itself. Integrin "activation" is a process by which integrin-ligand binding strength is increased and cell adhesion to ligand is modulated. Upon activation the platelet integrin $\alpha_{IIb}\beta_3$ is capable of binding fibrinogen leading to platelet aggregation. Likewise, activation of $\beta_2$ integrins on non-adherent leucocytes allows the rapid adhesion of these cells to ligand. Activation is mostly mediated by intracellular signals acting upon the integrin in a process termed "inside-out signalling" (Bazzoni and Hemler, 1998).

Two non-exclusive mechanisms effect integrin activation, affinity modulation and avidity modulation (Figure 1.5). Affinity modulation is simply explained as a conformational change within the integrin enabling ligand binding with a higher affinity. Avidity modulation is mediated by integrins clustering within the plane of the plasma membrane to generate concentrated areas of increased integrin activity. The relative contribution that each mechanism plays during integrin activation is integrin-dependent.

### 1.5.1 Integrin avidity modulation of $\beta_2$ integrins

Activation of the $\beta_2$ integrins is characteristic of avidity modulation with studies concentrating on LFA-1 ($\alpha_L\beta_2$). Leucocyte adhesion to endothelia, antigen presenting cells and cytotoxic T-cell target cells requires activation of LFA-1. Cross-linking of the T-cell receptor (TcR)/CD3 complex on T-cells stimulates LFA-1-mediated adhesion to ICAM-1 by rapid integrin clustering (Dustin and Springer,

1989), a process reproduced with phorbol ester treatment (Stewart and Hogg, 1996). Clustering of LFA-1 appears to be the dominant mechanism of TcR - and phorbol ester-stimulated adhesion. The actin cytoskeleton is critical for integrin clustering, since cytochalasin D (inhibits actin polymerisation) can inhibit integrin clustering and adhesion (van Kooyk and Figdor, 2000).

LFA-1 has also been observed to undergo affinity modulation upon divalent cation stimulation (Dransfield *et al.*, 1992; Stewart and Hogg, 1996) and Constantin *et al.* (2000) has shown that chemokines can induce the high affinity state in LFA-1. The chemokine induced high affinity state was suggested to be important during rapid lymphocyte arrest under flow conditions (Constantin *et al.*, 2000). Affinity modulation has also been observed in Mac-1 ($\alpha_M\beta_2$) (Altieri and Edgington, 1988).

### 1.5.2 Affinity modulation of integrins

Conformational changes attributed to affinity modulation of integrins have been observed in $\alpha_4\beta_1$, $\alpha_5\beta_1$, $\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_4\beta_7$ and $\alpha_{IIb}\beta_3$ (Altieri and Edgington, 1988; Crowe *et al.*, 1994; Dransfield *et al.*, 1992; Faull *et al.*, 1993; Masumoto and Hemler, 1993; Shattil *et al.*, 1985). Similar to LFA-1, crosslinking of TcR in a T lymphoid cell line increased adhesion, in this case mediated by an increase in $\alpha_5\beta_1$ affinity (Faull *et al.*, 1994). Integrin affinity modulation is best described for the $\alpha_{IIb}\beta_3$ integrin and platelet aggregation a physiological process dependent on affinity modulation.

Platelet aggregation in response to ADP was initially described to be dependent upon the rapid exposure of high affinity fibrinogen receptors on the surface of platelets in a $Ca^{2+}$ dependent manner (Bennett and Vilaire, 1979). Non-activated platelets fail to bind to fibrinogen; following stimulation activated platelets bind approximately 45,000-fibrinogen molecules/cell. These high affinity fibrinogen receptors were subsequently described as the GPIIb-IIIa glycoprotein and later identified as the $\alpha_{IIb}\beta_3$ integrin. Integrin clustering also contributes to the increase in fibrinogen binding and is required for the subsequent irreversible binding of ligand by $\alpha_{IIb}\beta_3$ (Hato *et al.*, 1998).

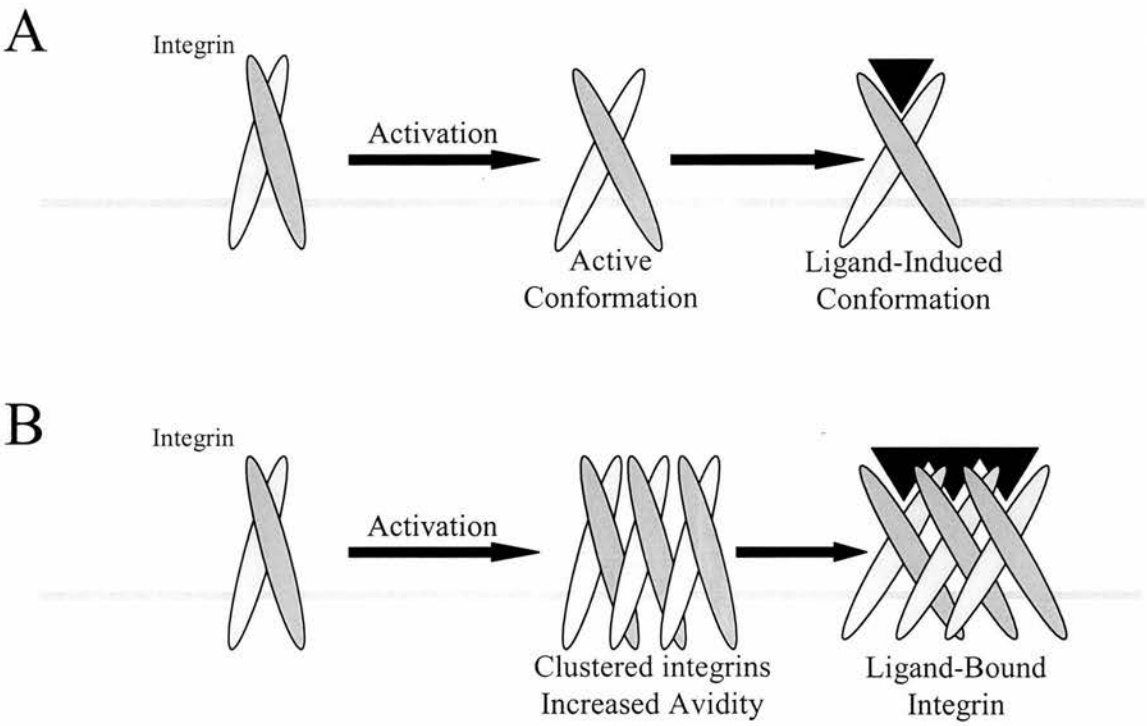


**Figure 1.5 Models of integrin activation.**

(A) Affinity modulation occurs through a conformational change that generates a high affinity ligand-binding integrin that is recognised by ligand-mimetic antibodies. Upon ligand binding, new antibody binding epitopes are revealed. (B) Avidity modulation occurs with clustering of integrins that generates a localised increase in ligand binding strength.

Shattil *et al.* (1985) described that following platelet activation, $\alpha_{IIb}\beta_3$ underwent a conformational change that generated the high affinity $\alpha_{IIb}\beta_3$ fibrinogen receptor. This receptor was specifically recognised by a monoclonal antibody called PAC1 (Shattil *et al.*, 1985). PAC1 is an IgMκ murine monoclonal antibody that mimics fibrinogen's ability to recognise the active integrin (ligand-mimetic antibody). The presence of an RGD sequence within the heavy chain of the PAC1 antibody enables affinity-specific recognition of $\alpha_{IIb}\beta_3$ (Kunicki *et al.*, 1996). PAC1 recognition of the active integrin was not dependent on the pentameric structure of the antibody (clustered integrins) as Fab fragments were still capable of recognising the active integrin (Abrams *et al.*, 1994).

## 1.6 Affinity modulation of $\alpha_{IIb}\beta_3$

Platelet agonists including thrombin, ADP, epinephrine and thromboxane $A_2$ can trigger affinity modulation of $\alpha_{IIb}\beta_3$ (Shattil *et al.*, 1998). The identical chemical structure of resting and activated $\alpha_{IIb}\beta_3$ suggests that phosphorylation, proteolytic cleavage and other post-translational modifications are not responsible for fibrinogen binding (Yan *et al.*, 2000). Upon thrombin stimulation an increase in resonance energy transfer between fluorochromes on the separate $\alpha_{IIb}$ and $\beta_3$ subunits was observed (Sims *et al.*, 1991). Changes in peptide mapping of $\alpha_{IIb}\beta_3$ also revealed that a conformational change within the integrin occurred upon activation (Yan *et al.*, 2000). In the absence of ternary structure information for $\alpha_{IIb}\beta_3$ the precise nature of the conformational change remains to be determined.

The conformational changes arising within $\alpha_{IIb}\beta_3$ following platelet activation allow ligand binding. Further conformational changes have also been shown to occur following ligand binding (Du *et al.*, 1991). Epitopes exposed on the integrin are recognised by a group of antibodies called anti-CLIBS (cation-and-ligand-influenced binding site) reflecting the cation and ligand sensitivity of these antibodies (Bazzoni and Hemler, 1998). These conformational changes may therefore be important for subsequent outside-in signalling mechanisms (Leisner *et al.*, 1999). Several of these

antibodies can promote ligand binding and platelet aggregation (e.g. LIBS6) by shifting the $\alpha_{IIb}\beta_3$ affinity equilibrium towards a high affinity state (Frelinger, III *et al.*, 1991).

Intracellular signalling activates $\alpha_{IIb}\beta_3$ and mutations within the cytoplasmic domains of $\alpha_{IIb}\beta_3$ have revealed areas important for integrin activation. Chinese Hamster Ovary (CHO) cells expressing $\alpha_{IIb}\beta_3$ were unable to bind to soluble fibrinogen or PAC1 even in the presence of platelet agonists. Anti-CLIB antibodies were able to stimulate PAC1 binding, indicating that this integrin was still capable of undergoing a conformational change (O'Toole *et al.*, 1990). The cytoplasmic domains of $\alpha_{IIb}$ and $\beta_3$ have been shown to interact (Ginsberg *et al.*, 2001) and interaction between subunits proximal to the transmembrane region is required to maintain the integrin in a low affinity state. This region-termed the "hinge-domain" can form a salt-bridge between the subunits (Hughes *et al.*, 1996) and is composed of a GFFKR and LLv-iHRD sequence in the $\alpha$ and $\beta$ subunits respectively (Figure 1.6). Deletion of the GFFKR region or mutation of residues within this domain leads to the constitutive activation of the integrin that is not inhibited by metabolic inhibitors. This region may therefore act as a physical "on-off" switch in determining integrin affinity (Ginsberg *et al.*, 2001; Hughes *et al.*, 1996; O'Toole *et al.*, 1991).

Unlike $\alpha_{IIb}\beta_3$, $\alpha_5\beta_1$ expressed in CHO cells was capable of binding ligand. CHO cells expressing $\alpha_{IIb}\beta_3$ chimeras containing the cytoplasmic domain of $\alpha_5\beta_1$ were constitutively active and capable of being bound by soluble fibrinogen and PAC1 (O'Toole *et al.*, 1991; O'Toole *et al.*, 1994). Activation was inhibited by metabolic inhibitors suggesting that intracellular signalling was responsible for the activation state of the chimera. The high affinity state of the chimera was determined by sequences within both $\alpha$ and $\beta$ subunits. Cytoplasmic domains of alpha subunits $\alpha_2$, $\alpha_{6A}$ and $\alpha_{6B}$ also conferred a high affinity while those from $\alpha_M$, $\alpha_L$ and $\alpha_V$ did not (O'Toole *et al.*, 1994).

Regions within the distal portion of the cytoplasmic domains are required for integrin activation (Figure 1.6). Amino acid substitutions of the $\beta$ subunit revealed that

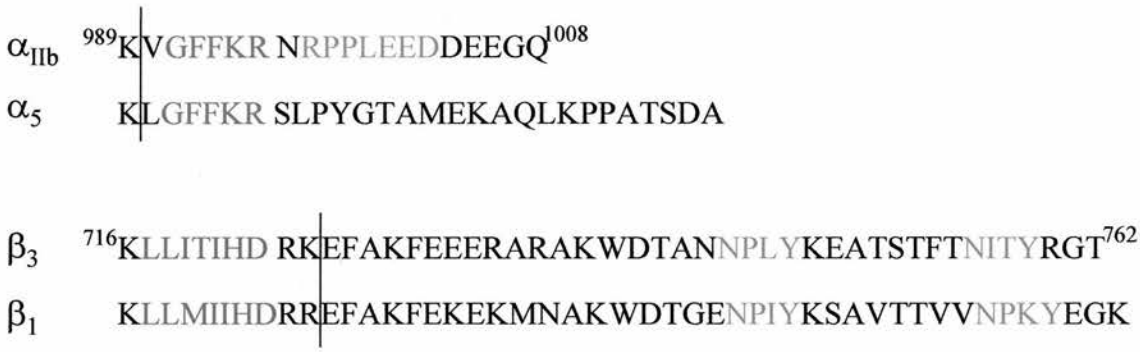


**Figure 1.6 Amino acid sequences of integrin cytoplasmic tails.**

Amino acid sequences of the integrin cytoplasmic tails are denoted as single letter amino acid code with superscript numbers denoting amino acid number. The positions of integrin chimeras are denoted by the straight line. The hinge sequences are shown in red and sequences important for integrin activation are shown in blue.

phosphorylation was not required for integrin activation. However, mutation of a conserved NPXY motif resulted in the complete loss in PAC1 binding from the chimeric-active integrin (O'Toole *et al.*, 1995). A similar mutation in $\beta_1$ was also shown to be important for $\beta_1$ activation (Mastrangelo *et al.*, 1999). Mutation of a second NXXY motif displayed a more modest reduction in PAC1 binding. These motifs, identified in several cell surface receptors, function as internalisation sequences; the β-turn structure within integrins may reflect a novel role for these sequences as recognition sites for intracellular proteins (Ginsberg *et al.*, 2001). The talin head domain has been shown to bind to this region and in CHO cells can activate $\alpha_{IIb}\beta_3$ affinity (Calderwood *et al.*, 1999). The β-turn structure of this region may be constrained by a RPPLEED sequence in the $\alpha_{IIb}$ subunit, thereby producing the inactive conformation (Ginsberg *et al.*, 2001).

Intracellular signalling is proposed to modulate integrin affinity through the binding of factors to the cytoplasmic tails. Overexpression of $\beta_1$ and $\beta_3$ in CHO cells expressing the active $\alpha_{IIb}\alpha_5\beta_3\beta_1$ chimera inhibited PAC1 binding. Titration of cytoplasmic factors away from the chimeric integrin to the isolated β tails is thought to mediate this inhibition (Chen *et al.*, 1994). Several proteins have been identified that can bind to integrins (reviewed in Liu 2000 and Hemler 1998) (Hemler, 1998; Liu et al., 2000). Proteins such as talin (Calderwood *et al.*, 1999), $\beta_3$-endonexin (Kashiwagi *et al.*, 1997), cytohesin-1 (Kolanus *et al.*, 1996) and CD98 (Fenczik *et al.*, 1997) have been shown to modulate integrin affinity in overexpression studies; the importance of the endogenous forms of these proteins remain to be clarified.

The intracellular signals modulating integrin affinity have yet to be fully characterised. Initial studies in platelets revealed that G-protein-coupled receptor signalling, phospholipase C stimulation and tyrosine phosphorylation were important in agonist-stimulated integrin activation (Shattil and Brass, 1987). Recent studies in cells other than platelets have shown that the Ras family of small GTP-binding proteins can also modulate integrin affinity (Hughes *et al.*, 1997). Inside-out signalling will be described in more detail below.

## 1.7 Consequences of Integrin Affinity on Cellular Processes

Modulation of integrin affinity alters integrin-ligand binding. Integrin-dependent processes such as adhesion, migration and matrix assembly can be sensitive to such changes, though they do not rely solely on ligand binding but also require post-occupancy events such as integrin clustering and cytoskeletal attachments.

CHO cells do not express $\alpha_{IIb}\beta_3$ and fail to spread on fibrinogen, whereas CHO cells that express $\alpha_{IIb}\beta_3$ spread on fibrinogen and also display random migration (Huttenlocher *et al.*, 1996). The migration rate is dependent on fibrinogen concentration and displays a bell-shaped correlation as described previously (Palecek *et al.*, 1997). Activation of $\alpha_{IIb}\beta_3$ with the LIBS6 mAb reduces the migration rate as a result of the increased adhesion to fibrinogen (Huttenlocher *et al.*, 1996). Deletion mutants within the integrin cytoplasmic tails can generate constitutively active integrins, the inability of cytoskeletal attachments however fail to alter the migration rate. (Ivins et al., 2000) has shown that neuronal outgrowth of late embryonic neuronal cells on laminin is dependent on the affinity of $\alpha_6\beta_1$. Suppression of $\alpha_6\beta_1$ during central nervous system development stops neuronal outgrowth from extending further; re-activating these integrins with $Mn^{2+}$ or R-Ras G38V restores neuronal outgrowth (Ivins *et al.*, 2000). The presence of an active $\alpha_V\beta_3$ on a subpopulation of metastatic breast cancer cells (but absent on non-metastatic cells) facilitates cell interactions with platelets, enabling flow arrest within the vasculature. Deletion of $\beta_3$ in these cells prevents interaction with platelets but may be restored by introducing the constitutively active $\beta_{3D723R}$ mutant (Felding-Habermann *et al.*, 2001). These breast cancer cells therefore express an active $\alpha_V\beta_3$ integrin to facilitate the metastatic process.

Fibronectin matrix assembly by cells also requires integrin-ligand binding. The $\alpha_{IIb}\beta_3$ integrin can bind fibronectin, but it cannot support matrix assembly in the inactive (low affinity) state; activating this integrin with LIBS6 mAb allows fibronectin matrix assembly (Wu *et al.*, 1995). The constitutively active integrin, $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ permits matrix assembly; suppressing this integrin with Ras G12V expression

abrogates matrix assembly by these cells (Hughes *et al.*, 1997). The fibrosarcoma tumour cell line HT1080 does not assemble a fibronectin matrix, but activating the endogenous $\alpha_5\beta_1$ integrin with $Mn^{2+}$ or a $\beta_1$ stimulating antibody restores matrix assembly by these cells. Inhibition of the elevated ERK1/2 activity in these cells due to an activated allele of N-Ras restored matrix assembly (Brenner *et al.*, 2000). It was therefore proposed that ERK signalling may suppress the $\alpha_5\beta_1$ integrin.

# Ras and Ras Signalling

The Ras proteins and associated signalling pathways have been the focus of intense research for the last twenty years. The Harvey and Kirsten strains of murine sarcoma retrovirus (Ha-MSV and Ki-MSV) were isolated from mouse tumours induced by the murine leukaemia virus that were passaged through rats (Harvey, 1964; Kirsten and Mayer, 1967). The transforming potential of these viruses was encoded by a 21kDa protein (Scolnick *et al.*, 1979; Shih *et al.*, 1979), which we now know as the viral oncogenes *ras*. The acronym Ras was derived from the words rat sarcoma, from which these genes were initially identified. The viral oncogenes from Ha-MSV and Ki-MSV were shown to be derived form normal rat cellular proteins called Harvey-Ras (Ha-Ras/H-Ras) and Kirsten-Ras (Ki-Ras/K-Ras) (Ellis *et al.*, 1981; Langbeheim *et al.*, 1980; Shih *et al.*, 1979). The importance of these *ras* genes was brought to the forefront upon the discovery that the dominant oncogenes of several human tumours were mutant alleles of the *ras* gene. These human oncogenes were shown to be homologous to the viral H-*ras* and K-*ras* (Der *et al.*, 1982; Parada *et al.*, 1982; Santos *et al.*, 1982). From a neuroblastoma cell line a third member of this family was identified, N-Ras (Shimizu *et al.*, 1983).

The *ras* genes were the first oncogenes implicated in human tumours and conversion of the normal cellular proto-oncogenes were mapped to single amino acid changes (Reddy *et al.*, 1982; Tabin *et al.*, 1982). Mutations in codons 12, 13 or 61 convert one of the three *ras* genes into active oncogenes present in a variety of human tumours (reviewed in Bos 1989) (Bos, 1989). In addition to the genesis of many tumours, oncogenic *ras* plays an essential role in tumour maintenance (Chin *et al.*, 1999).

## 1.8 Ras genes and proteins

The *ras* genes have been identified in most species ranging from yeast to humans. In fact the human *ras* gene can rescue a *ras*-deficient yeast cell and a mutant yeast Ras

protein can transform mouse fibroblasts (Defeo-Jones *et al.*, 1985; Kataoka *et al.*, 1985). The high degree of sequence conservation between species suggests that the *ras* genes play a fundamental role within the cell.

The *ras* genes contain four exons and a 5' non-coding exon (exon ϕ). The introns differ widely in their sequence and size, with the genomic sequences spanning 3, 7 and 35kb for H, N and K-*ras* respectively. K-*ras* has two alternative fourth exons producing K-*ras4*A and K-*ras4*B that differ only in their terminal 25 amino acids (Barbacid, 1987). These genes encode for 189 amino acids (188 for K-*ras4*B) and the proteins are all approximately 21kDa in size, hence the name p21 Ras. The *ras* promoter sequence is characteristic of cell housekeeping genes with the genes being expressed in all cell lineages and organs.

Sequence comparison of the Ras proteins reveal that the first 86 amino acids are identical and the next 78 reveal a very high degree of homology. It is within the terminal 25 amino acids the "hypervariable domain" that the sequence is divergent. The *ras* genes code for proteins that bind guanine nucleotides and have an intrinsic GTPase activity (Gibbs *et al.*, 1984; McGrath *et al.*, 1984; Shih *et al.*, 1980; Sweet *et al.*, 1984). Studies have shown that Ras complexed with guanosine triphosphate (GTP) is active and that hydrolysis of GTP to GDP produces an inactive Ras-GDP protein (Field *et al.*, 1987; Sweet *et al.*, 1984). Scheele *et al.* (1995) have shown that of the 20,000 Ras molecules, in a resting NIH 3T3, cell approximately 0.3% is in the active GTP-bound state (Scheele *et al.*, 1995). The mechanism by which endogenous Ras activity is increased will be described shortly.

Ras proteins are synthesised on free ribosomes within the cytoplasm (Ulsh and Shih, 1984). Following synthesis a series of post-translational modifications at the C-terminal CAAX motif target Ras to the plasma membrane (Figure 1.7). Cysteine$^{186}$, conserved in all Ras proteins, is farnesylated, the AAX sequence removed by proteolysis and the Cys$^{186}$ carboxylmethylated. Palmitoylation of cysteines in H, N-Ras and K-Ras4A or a poly-lysine stretch in K-Ras4B further contributes to the membrane localisation signal of the Ras proteins (Hancock *et al.*, 1990). Membrane localisation is essential for Ras function and that deletion of Cys$^{186}$ prevents Ras

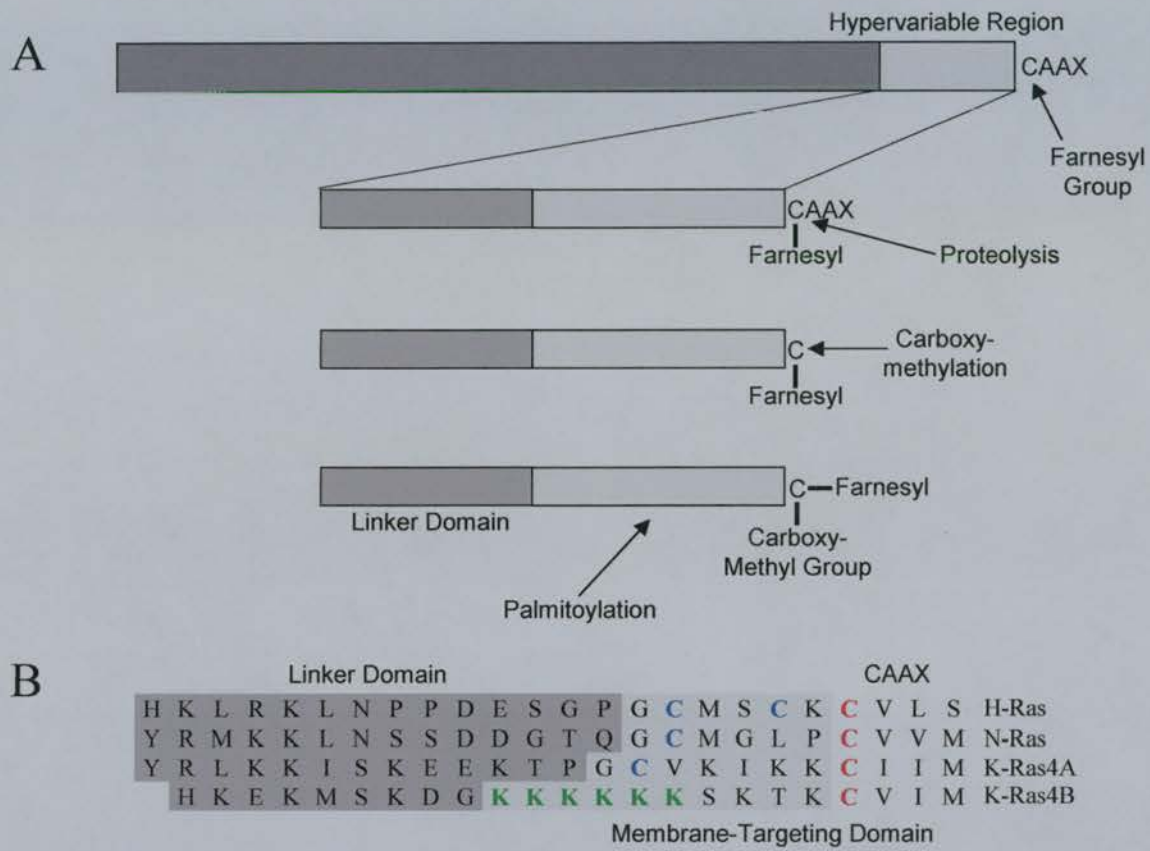


**Figure 1.7 Ras C-Terminal Modifications.**

(A) An expanded view of the Ras hypervariable region (grey) shows the sequential modifications that occur to newly translated Ras proteins. (B) Sequence alignment of the four Ras isoforms. The CAAX motif (light grey) contains the conserved cysteine residue (red). Cysteines that are palmitoylated are shown in blue with the polybasic lysine residues of K-Ras4B shown in green. Modified from Prior *et al.* (2001).

transforming activity (Willumsen *et al.*, 1984). Membrane targeting of such mutants by other lipid modifications can rescue the transforming capacity of the Ras protein (Buss *et al.*, 1989; Lacal *et al.*, 1988).

Mutation and deletion studies have revealed several regions important for Ras function (Figure 1.8). Guanine nucleotide binding is mediated by interactions with both the phosphate groups and the purine ring. Sequence motifs important for guanine nucleotide binding show similarity with other GTP/GDP binding proteins, including heterotrimeric G-proteins and elongation factor-Tu (EF-Tu) (Lowy and Willumsen, 1993). The intrinsic GTPase activity is contained within the conserved N-terminal 166 amino acids and mutation of residues 12 or 61 disrupt the catalytic rate of hydrolysis (Sweet *et al.*, 1984). Structural predictions suggest that amino acid substitution of $Gly^{12}$ may disrupt the interaction with the γ-phosphate thereby reducing GTP hydrolysis (Barbacid, 1987). The hypervariable domain is one of five regions that are dispensable for cell transformation. However, recent studies show that this region may be important for correct membrane micro-domain localisation and effector pathway utilisation (Booden *et al.*, 2000; Jaumot *et al.*, 2001; Prior and Hancock, 2001).

Mutations within the "effector" domain drastically reduce Ras transforming ability without affecting guanine nucleotide binding or hydrolysis (Sigal *et al.*, 1986; Willumsen *et al.*, 1986). This region, also known as "switch 1", undergoes conformational changes upon GTP binding and hydrolysis (Pai *et al.*, 1989; Schlichting *et al.*, 1990), the dynamics of which have recently been described (Ma and Karplus, 1997). The effector domain has been shown to be essential in binding the known Ras effectors (Rodriguez-Viciana *et al.*, 1997; White *et al.*, 1995).

### 1.8.1 Differences between the Ras isoforms

The Ras proteins are virtually identical except for the hypervariable region. The strong conservation of these divergent sequences between species suggests the importance of these unique sequences (Barbacid, 1987). Although the *ras* genes are expressed ubiquitously, each gene has a slightly different expression pattern between

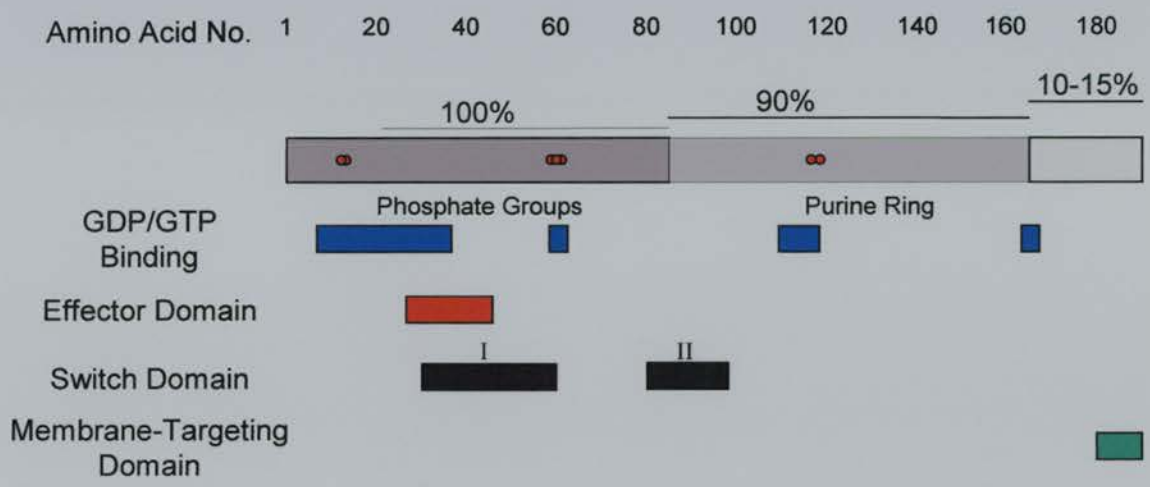


**Figure 1.8 Schematic of the structure of Ras.**

The Ras protein is divided into three regions according to the percentage sequence homology between the Ras isoforms. The individual regions important for Ras functions are highlighted with the coloured blocks. Red circles denote positions of activating mutants.

organs: H-*ras* is highly expressed in the brain, N-*ras* in the thymus and testis and K-*ras* in the gut and testis (Leon *et al.*, 1987). These expression patterns, however, do not correlate with the selective activation of the *ras* genes in different tumours (Fiorucci and Hall, 1988). For instance K-*ras* is preferentially activated in colon and pancreas carcinomas, H-*ras* in bladder and kidney carcinomas and N-*ras* in myeloid and lymphoid disorders (Bos, 1989).

Of the three *ras* genes only K-*ras* appears to be essential and sufficient for development, as double knockout of H-*ras* and N-*ras* are viable (Esteban *et al.*, 2001; Koera *et al.*, 1997; Umanoff *et al.*, 1995). Effector pathway utilisation also varies between isoforms: K-Ras is a more potent activator of Raf than H-Ras, but the converse is true for PI3-kinase activation (Yan *et al.*, 1998). The Ras isoforms also differ in their ability to induce cell transformation and migration (Voice *et al.*, 1999). The localisation of these Ras isoforms into different plasma membrane micro-domains may contribute to their differing biological effects (Prior and Hancock, 2001).

## 1.9 Regulation of Ras activity

The Ras proteins cycle between an active GTP-bound and an inactive GDP-bound state (Figure 1.9) (Bourne *et al.*, 1990). The majority of Ras within a cell is in the GDP-bound state (Scheele *et al.*, 1995) and therefore activation requires replacement of GDP by GTP. This exchange of GTP for GDP requires proteins known as Guanine Exchange Factors (GEF) such as SOS (Quilliam *et al.*, 1995). These GEF proteins form a complex with Ras-GDP and GDP is displaced, being immediately replaced by GTP due to the high GTP/GDP ratio within a cell. The GEF subsequently dissociates, leaving an active Ras-GTP complex (Boguski and McCormick, 1993). GTP is hydrolysed back to GDP by the intrinsic GTPase activity, recycling Ras back to the inactive state. The intrinsic rate of GTP dissociation is very slow (John *et al.*, 1990), therefore GTPase Activating Proteins (GAP) stimulate GTP hydrolysis, thereby limiting Ras activity (Polakis and McCormick, 1993).

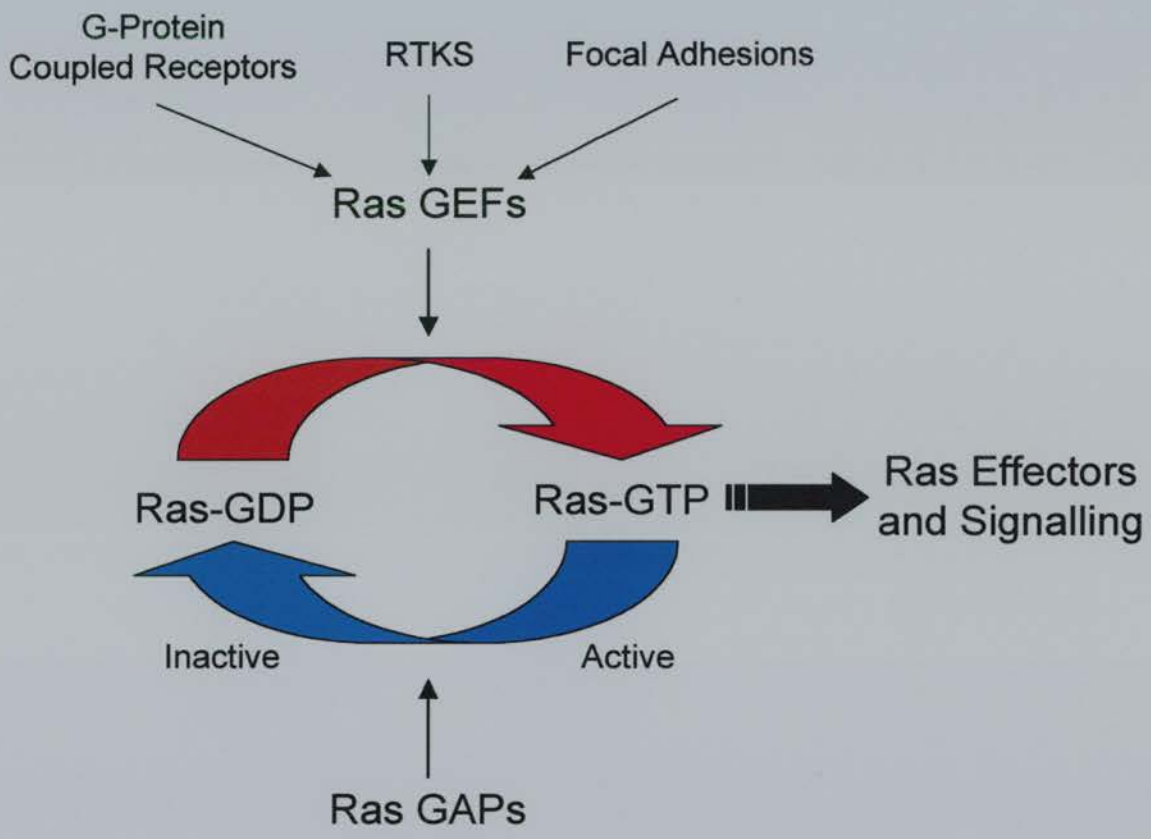


**Figure 1.9 Cycling of Ras between GDP and GTP bound states.**

Ras GEFs can stimulate the exchange of bound GDP for GTP producing the active Ras protein. Ras GAPs stimulate the intrinsic Ras GTPase activity that hydrolyses GTP to GDP, returning Ras to the inactive state.

### 1.9.1 Ras activation

Ras is the convergence point for extracellular signals to be transmitted into the cell. Receptors mediating Ras activation include receptor tyrosine kinases (e.g. epidermal growth factor (EGF) receptor), cytokine receptors (e.g. IL-2 receptor), T-cell receptors, G-protein coupled receptors (e.g. muscarinic receptor) and integrins.

The best described of these signals is that of growth factor stimulation of Ras activity. Platelet derived growth factor (PDGF) can increase the proportion of Ras-GTP within minutes of treatment (Gibbs *et al.*, 1990). Receptor tyrosine kinases (RTK) autophosphorylate several key tyrosine residues within their cytoplasmic region upon ligand binding (Pazin and Williams, 1992). These phosphotyrosyl sites allow binding, through SH2 domains, of adaptor proteins including Shc and Grb2 (Baltensperger *et al.*, 1993; Gale *et al.*, 1993; Lowenstein *et al.*, 1992). Autophosphorylation of Shc upon RTK activation can also generate a Grb2 binding site (Rozakis-Adcock *et al.*, 1992). The transient activation of Ras is mediated by recruitment of the Ras-GEF SOS to the plasma membrane via a SOS/Grb2 complex. This may be mediated either by direct binding of Grb2 to RTK or via Shc (Li *et al.*, 1993; Olivier *et al.*, 1993). Integrin activation of Ras at focal adhesion has also been described previously to use the Shc/Grb2 complex (Figure 1.3).

G-protein coupled receptors activate Ras via their associating heterotrimeric G-protein subunits. G-protein subunits can mediate phosphorylation and transactivation of RTK in a growth factor-independent manner, thereby recruiting the adaptor molecules as described above (Della Rocca *et al.*, 1999; Luttrell *et al.*, 1999). G-proteins can also use the integrin-signalling complex to activate Ras (reviewed in Luttrell 1999). Gβγ subunits can also activate PI3-kinase γ (Lopez-Ilasaca *et al.*, 1997; Malarkey *et al.*, 1995; van Biesen *et al.*, 1995) and in *xenopus*, expression of the p110 subunit of PI3-kinase produced an increase in GTP-loading of Ras (Hu *et al.*, 1995a). G-proteins are also capable of activating Ras-GEFs such as RasGRF1 and RasGRP (Reuther and Der, 2000). Gα subunits have also been shown to increase ERK1/2 activation in a Ras-independent manner mediated by PKC activation

(Gudermann *et al.*, 2000) or through the activation of Rap1GEFs that can activate B-Raf (Mochizuki *et al.*, 1999).

T-cell activation of Ras also uses the Grb2/SOS complex to activate Ras; membrane recruitment of Grb2 may be mediated by protein-interactions with T-cell proteins such as SLP-76 or LAT (Marie-Cardine and Schraven, 1999)

Mammalian SOS is related to the *drosophila* son-of-sevenless product required for Ras activation in *drosophila* (Bowtell *et al.*, 1992). SOS contains a cdc25 homology domain that is responsible for GEF activity (Lai *et al.*, 1993); it also contains several interaction domains including SH3, Dbl homology and pleckstrin homology domains (Malumbres, 1998). Other Ras-GEFs include RasGRF, RasGRF2 and RasGRP (Ebinu *et al.*, 1998; Fam *et al.*, 1997; Martegani *et al.*, 1992).

### 1.9.2 Ras inactivation

Ras-GAPs stimulate the intrinsic GTPase activity of Ras, thereby reducing Ras activity. Several of the oncogenic mutants of Ras such as G12V are unresponsive to GAP activity and are therefore constitutively in the active GTP-bound state (Scheffzek *et al.*, 1997; Trahey and McCormick, 1987). Several mammalian GAPs have been identified (Malumbres, 1998) including p120-GAP (Trahey and McCormick, 1987) and neurofibromatosis type 1 protein (NF1) (Ballester *et al.*, 1990). Whether these proteins also have Ras effector function is described in Chapter4.

## 1.10 Ras Superfamily

The Ras proteins were the first described members of the Ras superfamily of small GTP-binding proteins, which now contains more than 100 members (reviewed in Takai 2001). According to their primary sequence and biological function these members can be classified into sub-families: Ras, Rho, Rab, Arf and Ran (Matozaki *et al.*, 2000; Takai *et al.*, 2001). The Rho family members are associated with the cell cytoskeleton and gene expression (Mackay and Hall, 1998; Ridley and Hall, 1992) and the Rab family with secretory and endocytic pathways (Schimmoller *et al.*,

1998). The Arf family are also associated with vesicular trafficking pathways and can also activate phospholipase D (Brown *et al.*, 1993), (Mitchell *et al.*, 1998; Moss and Vaughan, 1998) while the Ran family are key for coordinating and driving nuclear traffic (Moore, 1998).

The Ras subfamily currently contains 16 members: H-Ras, N-Ras, K-Ras4A and K-Ras4B, R-Ras proteins (R-Ras, R-Ras2/TC21 and R-Ras3/M-Ras), Ral proteins (A and B), the Raps (1A, 1B, 2A and 2B), Rheb, Rin (this should not be confused with Rin1, a Ras effector) and Rit (Matozaki *et al.*, 2000). With the exception of the Ras proteins, Rap2A, Rin and Rit which all have a farnesyl group, all are localised to the membrane by a geranylgeranyl lipid group, which may allow specific membrane targeting for their biological properties (Reuther and Der, 2000).

R-Ras is more than 50% identical to Ras with an identical effector domain (Lowe *et al.*, 1987). R-Ras can bind to the same effectors as Ras, but it has only been reported to activate PI3-kinase (Marte *et al.*, 1997) and although it has been shown to transform cells, it does so to a lesser extent than Ras (Cox *et al.*, 1994). R-Ras2/TC21 also shows homology to Ras (Drivas *et al.*, 1990) and transforming ability (Graham *et al.*, 1994) and has been the only other Ras member found to be mutated in a human tumour cell line (Chan *et al.*, 1994). R-Ras may have a role in apoptosis (Wang *et al.*, 1995) and integrin affinity regulation (Sethi *et al.*, 1999; Zhang *et al.*, 1996a) but the precise function of the R-Ras family remains to be clarified.

The Rap proteins, initially proposed to antagonise Ras biological activity (Kitayama *et al.*, 1989), may play a more complex role in cell signalling (Reuther and Der, 2000). The Ral proteins are activated by RalGEFs, including RalGDS, that are known Ras effectors (see below). Biological activity of recent additions to the Ras family, Rheb (Yamagata *et al.*, 1994) and Rit (Lee *et al.*, 1996), remains to be determined (Reuther and Der, 2000).

The identification of several exchange factors and GTPase-activating proteins for many of the Ras subfamily members suggests that each may have specific biological

roles within the cell. Elucidation of effectors downstream of these proteins may allow a better understanding of the signalling pathways initiated by these proteins.

## 1.11 Ras signalling and effectors

Ras is the convergence point for a multitude of extracellular activating signals. The intricacy and complexity of Ras signalling continues with its downstream pathways. Ras has long been known to mediate cell cycle progression, most evident in transformed cells; several other biological effects are also attributed to Ras signalling including cell survival, morphogenesis, differentiation and migration. The divergent biological effects attributed to Ras activation are in part dependent upon the downstream pathways activated by the Ras "effectors". Ras effectors preferentially bind to the Ras-GTP complex, display an increase in activity following Ras interaction and are sensitive to mutations within the Ras effector domain (Marshall, 1996). One of the first and most intensely studied Ras effector is Raf.

As with the discovery of the *ras* genes, *raf* was identified as the transforming component of the mouse sarcoma virus 3611 (Rapp *et al.*, 1983). The pro-oncogene *c-raf-1* was identified in non-transformed cells (Kozak *et al.*, 1984) and was shown to be capable of activating the p42/p44 mitogen activated protein kinases (MAPKs) or extracellular-signal-regulated kinases (ERK1/2) (Howe *et al.*, 1992). Activation of ERK1/2 observed following Ras activation was dependent on MEK activation (Kosako *et al.*, 1992; Kyriakis *et al.*, 1992). Raf was capable of phosphorylating and activating MEK, placing Raf upstream of MEK and ERK1/2 in the Ras mitogenic signalling pathway (Avruch *et al.*, 1994). The discovery that Raf is an effector of Ras (Han *et al.*, 1993; Warne *et al.*, 1993; Zhang *et al.*, 1993) suggested that a signalling cascade of Ras-Raf-MEK-ERK was required for mitogenic signalling (Avruch *et al.*, 1994; Dickson *et al.*, 1992; Gomez and Cohen, 1991). This signalling cascade will be described in more detail shortly.

Historically there has been a considerable body of evidence supporting the hypothesis that the Raf-ERK cascade was solely responsible for Ras function. Constitutively active mutants of Raf and MEK were both able to transform rodent

fibroblasts similar to that of Ras (Alessi *et al.*, 1994; Bonner *et al.*, 1985; Mansour *et al.*, 1994; Stanton, Jr. *et al.*, 1989) and that Raf can overcome the loss of Ras function to transform cells (Feig and Cooper, 1988; Smith *et al.*, 1986a). Expression of dominant negative mutants of Raf-1, MEK and ERK can attenuate Ras transforming activity (Bruder *et al.*, 1992; Cowley *et al.*, 1994; Khosravi-Far *et al.*, 1995; Kolch *et al.*, 1991; Schaap *et al.*, 1993; Westwick *et al.*, 1994). Genetic studies performed in *drosophila* and *C.elegans* also revealed the importance of the Ras-Raf pathway (Campbell *et al.*, 1998; Han *et al.*, 1993).

The increasing number of Ras effectors identified indicates that Raf signalling may be part of a far more intricate Ras signalling pathway. Ral-GEFs and PI3-kinase are just two of the best described alternative Ras effectors (Malumbres, 1998). Figure 1.10 describes the known Ras effectors and a simplified schematic of their downstream pathways. This growing number of Ras effectors suggests that Raf may not be the sole effector responsible for Ras function.

Ras and Raf are both capable of fully transforming NIH 3T3 rodent fibroblasts. Similar experiments performed in rat intestinal epithelial (RIE-1) cells show that only activated Ras can cause transformation and that Raf-independent signalling was required for transformation (Oldham *et al.*, 1996). In human thyroid epithelial cells activated MEK upregulated ERK1/2 activation; but only activated Ras led to proliferation (Gire *et al.*, 1999). Alternative signalling pathways have also been observed in yeast homologues of Ras signalling (Campbell *et al.*, 1998). The most direct evidence for Raf-independent pathways comes from the generation of Ras effector mutants. White *et al.* (1995) generated Ras G12V effector mutants that displayed differential binding and activation of Raf, RalGDS and PI3-kinase (Rodriguez-Viciana *et al.*, 1997; White *et al.*, 1995). The interaction of these effector mutants with Ras effectors is summarised in Table 1.2. Ras (G12V, T35S), capable of binding Raf, display limited transforming ability; co-expression with Ras mutants defective for Raf binding produced synergistic transforming ability (Khosravi-Far *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997; White *et al.*, 1995). Ras-dependent membrane ruffling observed in quiesced fibroblasts was only observed with the Ras

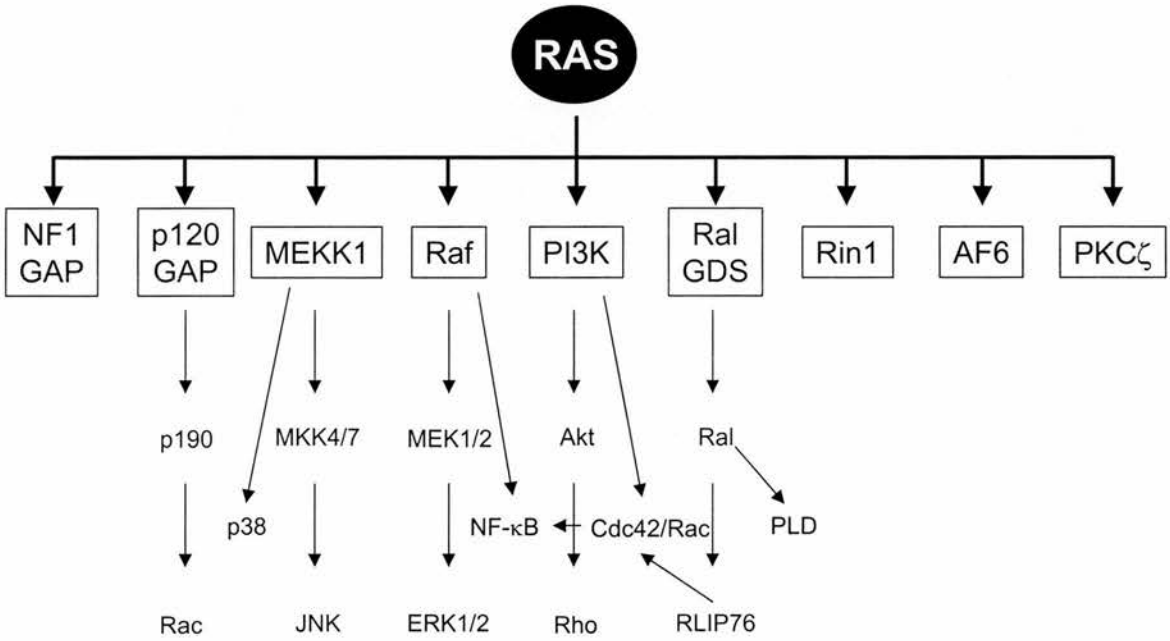


**Figure 1.10 Ras Effector Pathways.**

Identified Ras effectors (boxed) can activate several downstream pathways that mediate the multitude of Ras functions.

**Table 1.2 Ras Effector Mutant Interactions.**

| | Raf | RalGDS | p110α | Rin-1 | PLCε | AF6 | PKCζ | MEKK1 |
|---|---|---|---|---|---|---|---|---|
| Ras G12V | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| Ras (G12V, E37G) | ✖ | ✓ | ✖ | ✓ | ✓ | ✓ | nd | nd |
| Ras (G12V, T35S) | ✓ | ✖ | ✖ | ✖ | ✖ | ✓ | nd | nd |
| Ras (G12V, Y40C) | ✖ | ✖ | ✓ | ✖ | ✖ | ✓ | nd | nd |

Interactions between Ras effector mutants and effectors denoted by ✓; ×- no interaction observed; nd - not determined.

(G12V, Y40C) mutants and not with activated Raf (Joneson *et al.*, 1996). Khosravi-Far *et al.* (1996) showed that all three Ras mutants were capable of inducing growth in low serum, soft agar and the formation of tumours in nude mice (Khosravi-Far *et al.*, 1996); however; only the Ras (G12V, T35S) mutant has been shown to display experimental metastasis *in vivo* (Webb *et al.*, 1998). Biological effects attributed to Ras may therefore require a combination of signals through the Ras effector pathways.

### 1.11.1 Ral guanine exchange factors

Ral-GEFs are Ras effectors that activate the Ras related proteins of the Ral family. Four Ral-GEFs have been identified, Ral guanine nucleotide dissociation stimulator (RalGDS) (Albright *et al.*, 1993; Hofer *et al.*, 1994; Spaargaren and Bischoff, 1994), RalGDS-like1/2 (Rgl1/2) (Hill *et al.*, 1995; Peterson *et al.*, 1996) and RalGDS-like factor (Rlf) (Wolthuis *et al.*, 1996).

The Ras (G12V, E37G) mutant capable of binding the RalGEFs produced synergistic transforming ability in combination with Raf signalling (Khosravi-Far *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997; White *et al.*, 1996). The Ras (G12V, E37G) mutant has also been shown to have effects on proliferation of thyrocytes (Miller *et al.*, 1997) and synergised with Ras (G12V, Y40C) in growth factor-independent myeloid cell growth (Matsuguchi and Kraft, 1998). Synergism in cell transformation with Raf signalling has also been observed with RalGDS and Rlf (Urano *et al.*, 1996; White *et al.*, 1996; Wolthuis *et al.*, 1997) and these GEFs can also reproduce the Ras G12V effects on transcription (Ramocki *et al.*, 1998; Wolthuis *et al.*, 1997). The significance of RalGEF-Ral activity has been highlighted by the ability of a dominant negative RalA (28N) to inhibit Ras function (Miller *et al.*, 1997; Urano *et al.*, 1996; White *et al.*, 1996; Wolthuis *et al.*, 1997). The GTPase defective RalA mutant (23V) has in general failed to reproduce any Ras function and it has been proposed that hydrolysis of GTP may be important for Ral effector activation (Wolthuis *et al.*, 1997). RalGEFs and Ral signalling may therefore contribute to Raf-independent Ras function.

A putative Ral effector, RLIP76 (Ral-binding domain) has been identified that displays GAP activity towards Rac1 and Cdc42 (Cantor *et al.*, 1995; Jullien-Flores *et al.*, 1995). RLIP76 may therefore allow crosstalk between the Ral and Rac/Cdc42 pathways downstream of Ras. Ral is also required for phospholipase D (PLD) activation in forming a complex between Ral-PLD and Arf (Jiang *et al.*, 1995; Luo *et al.*, 1998). How these effectors relate to Ral function remains to be clarified.

### 1.11.2 PI3-kinase

Phosphatidylinositol-3-OH kinase (PI3-kinase) phosphorylates the D3 position of the inositol ring on phosphoinositides (Carpenter and Cantley, 1996). Both Ras G12V and Ras (G12V, Y40C) can stimulate PI3-kinase activity (Rodriguez-Viciana *et al.*, 1994; Rodriguez-Viciana *et al.*, 1997) and the subsequent increase in phosphorylated lipid products. With the use of Ras (G12V, Y40C) and activated mutants of PI3-kinase, PI3-kinase activity has been shown to contribute to Ras mediated transformation, DNA synthesis, cell morphology and cell survival (Kauffmann-Zeh *et al.*, 1997; Khosravi-Far *et al.*, 1996; Matsuguchi and Kraft, 1998; Rodriguez-Viciana *et al.*, 1997; Xue *et al.*, 2000). Phosphorylated lipid products of PI3-kinase can regulate the activities of a wide range of proteins (Downward, 1998); Akt, p70S6 kinase and RacGEFs have received the most attention. Akt activity modulates glycogen metabolism, protein synthesis and cell survival (Datta *et al.*, 1999). The anti-apoptotic effects of Akt are mediated by actions on the pro-apoptotic factor BAD, caspase 9 and NF-κB signalling (Datta *et al.*, 1999). Activation of RacGEFs by PI3-kinase products (Hawkins *et al.*, 1995) may link Ras signalling to the Rho family of proteins and mediate the cytoskeletal changes observed in Ras transformed cells (Malumbres, 1998).

The Rho family of proteins has profound effects upon the actin cytoskeleton (Ridley and Hall, 1992) and can promote cell cycle progression (Olson *et al.*, 1995). Rac and Cdc42 can stimulate JNK and p38 MAPK activity (Coso *et al.*, 1995; Westwick *et al.*, 1994) and all three members can activate transcription factors (Hill *et al.*, 1995). Khosravi-Far *et al.* (1995) and other groups have reported that activated mutants of

these proteins synergise with Raf in mediating cell transformation and that dominant negative mutants reduce Ras transforming activity (Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995). These and other results show that the Rho proteins may contribute to Ras biological function (Bar-Sagi and Hall, 2000).

### 1.11.3 Other Ras Effectors

Ras signalling research has concentrated on the three effectors Raf, RalGEFs and PI3-kinase. Several other Ras effectors have been identified: MEKK1 (Russell *et al.*, 1995), Phospholipase C-ε (Kelley *et al.*, 2001; Lopez *et al.*, 2001; Song *et al.*, 2001), p120-GAP (Leblanc *et al.*, 1998), AF6 (Kuriyama *et al.*, 1996), Rin1 (Han and Colicelli, 1995) and PKCζ (Diaz-Meco *et al.*, 1994). Ras-mediated signalling and biological effects attributed to these effectors are described further in Chapter 4.

## 1.12 Raf Signalling

Raf, a serine/threonine kinase, couples Ras activation to mitogenic signalling by ERK1/2 (Figure 1.11). Considerable effort has been directed toward the understanding of the mechanisms leading to Raf activation and subsequent activation of ERK1/2. Three Raf isoforms have been identified in mammals, Raf-1 (c-Raf-1), A-Raf and B-Raf). Pritchard *et al.* (1995) have shown that conditionally oncogenic forms of the Raf isoforms display differential catalytic activity towards MEK (B-Raf > Raf-1 > A-Raf) and that this activity is represented by changes in ERK activation (Pritchard *et al.*, 1995). The Raf isoforms display differential expression patterns within mice and knockout studies reveal that each isoform may have unique functions (Hagemann and Rapp, 1999; Wojnowski *et al.*, 1997; Wojnowski *et al.*, 1998; Wojnowski *et al.*, 2000).

### 1.12.1 Raf Activation

Upon Ras activation, Raf is recruited to the plasma membrane where it becomes activated. Targeting Raf to the plasma membrane generates an active Raf protein (Stokoe *et al.*, 1994). Raf contains two functional domains, an amino-terminal

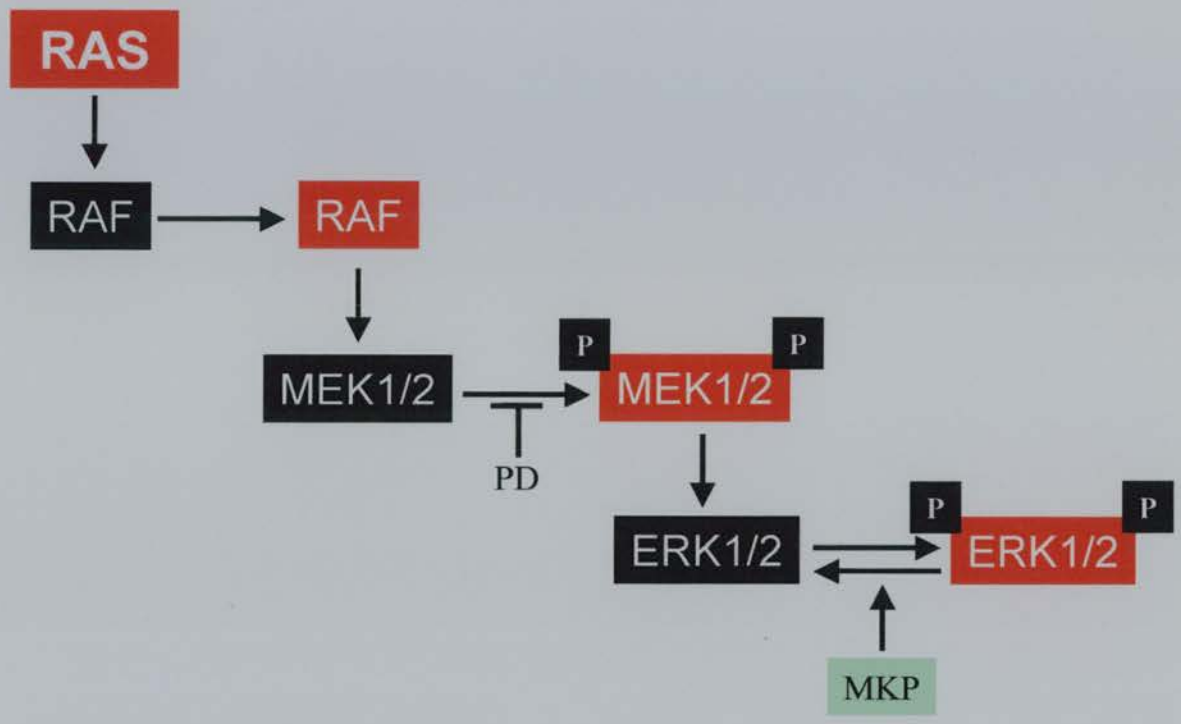


**Figure 1.11 Ras-ERK Cascade.**

The Ras-ERK signalling cascade is shown with active Ras (red box) activating inactive Raf (black box). This cascade continues with phosphorylation of MEK and subsequently ERK1/2. Map Kinase Phosphatase (MKP) can dephosphorylate ERK1/2 returning it to the inactive state. PD – PD098059 binds inactive MEK1 to cause inhibition.

regulatory domain and the carboxy-terminal catalytic domain. Two Ras binding motifs, the Ras binding domain (RBD) and a cysteine-rich domain (CRD), are present in the amino-regulatory domain (Morrison and Cutler, 1997). The amino-terminal suppresses catalytic activity and its removal, forming Raf-BxB, constitutively activates Raf (Stanton, Jr. *et al.*, 1989). Inactive Raf exists within the cytosol complexed with several proteins, including the phosphoserine binding protein 14-3-3 (Wartmann and Davis, 1994). This complex is recruited to the plasma membrane by an initial interaction between active Ras and the Raf RBD. A possible conformational change within Raf, facilitated by phosphatidylserine (PS) and displacement of the 14-3-3 proteins, permits Ras binding independent of Raf RBD (Hu *et al.*, 1995b) to Raf CRD. Phosphorylation of $Ser^{338}$ and $Tyr^{340/341}$ by Pak (Chaudhary *et al.*, 2000) and oncogenic Src (Marais *et al.*, 1995) respectively leads to full activation of Raf kinase activity. Raf catalytic activity is also modulated by phosphorylation by protein kinase A (PKA) (Wu *et al.*, 1993), Akt (Zimmermann and Moelling, 1999) and protein kinase C (PKC) (van Dijk *et al.*, 1997).

Most of the studies relating to Raf activation have concentrated on Raf-1; whose full activation requires Ras-mediated membrane localisation and phosphorylation (Blumer and Johnson, 1994; Marais *et al.*, 1995). A-Raf displays a similar requirement for membrane localisation and phosphorylation for activation. In contrast, B-Raf is strongly activated by Ras alone (Marais *et al.*, 1997). Activation of B-Raf can also occur through an interaction with active Rap. The activation of Rap GEFs leads to the GTP loading of Rap and subsequent activation of B-Raf (Bos, 1998). B-Raf activation produces an increase in ERK1/2 activation that is independent of Ras activity (Ohtsuka *et al.*, 1996).

### 1.12.2 Raf to ERK Signalling

Raf to ERK1/2 signalling described in Figure 1.11, requires the activation of the dual specificity kinases MAPK/ERK Kinase1/2 (MEK1/2) (Dent *et al.*, 1992), also known as Map Kinase Kinase1/2 (MAPKK1/2) (Dhanasekaran and Premkumar, 1998). MEK1/2 are cytoplasmic proteins of 43kDa and 46kDa respectively and are encoded

by two distinct genes on separate chromosomes (Brott *et al.*, 1993; Zheng and Guan, 1993).

Raf, phosphorylates both $Ser^{218}/Ser^{222}$ on MEK1 and $Ser^{219}/Ser^{223}$ on MEK2 (Alessi *et al.*, 1994) leading to full activation of MEK activity. Substitution of both of these serine residues to either glutamic or aspartic acid residues generated constitutively active mutants (Alessi *et al.*, 1994; Cowley *et al.*, 1994; Mansour *et al.*, 1994), capable of transforming NIH 3T3 cells *in vitro* (Cowley *et al.*, 1994; Mansour *et al.*, 1994). The interaction between Raf and MEK can be disrupted by Raf Kinase inhibitor protein (RKIP), an adaptor protein that binds both Raf and MEK and prevents MEK activation. This inhibition reduces Raf induced gene expression and transformation of NIH 3T3 cells (Yeung *et al.*, 1999). The regulation of RKIP binding may therefore place a threshold level of Raf activity required prior to MEK interaction and activation (Kolch, 2000).

MEK, identified by its ability to activate ERK (Crews *et al.*, 1992; Crews and Erikson, 1992; Nakielny *et al.*, 1992), phosphorylates ERK on threonine and tyrosine residues. These residues, $Thr^{183}$ and $Tyr^{185}$ (ERK2) are present within the MAPK consensus sequence of Thr-X-Tyr, where X is glutamic acid (TEY motif) (Payne *et al.*, 1991; Ray and Sturgill, 1988). Phosphorylation of both of these residues is required for activation (Anderson *et al.*, 1990) and stimulates ERK activity by 500-1000 fold compared to dephosphorylated ERK (Robbins *et al.*, 1993). Dephosphorylation of these residues returns ERK1/2 back to its inactive state. Map kinase phosphatases (MKP) that can specifically dephosphorylate both threonine and tyrosine residues can regulate *in vivo* ERK activity (Alessi *et al.*, 1993; Keyse, 1998). Mimicking the negative charges on these residues with glutamic or aspartic acid residues have failed to generate constitutively active mutants (Her *et al.*, 1993; Robbins *et al.*, 1993). An in-frame fusion between ERK2 and MEK1 produced a constitutively active ERK2 fusion protein that had no effect on endogenous ERK activity (Robinson *et al.*, 1998).

ERK2 was initially identified as a protein phosphorylated during growth factor stimulation (Ahn *et al.*, 1991; Boulton *et al.*, 1991; Rossomando *et al.*, 1989).

Activation of ERK is essential for Ras-mediated mitogenic signalling. Upon activation, ERK can translocate into the nucleus (Chen *et al.*, 1992; Lenormand *et al.*, 1993; Sanghera *et al.*, 1992) where several ERK1/2 substrates have been identified (Su and Karin, 1996). Phosphorylation of the transcription factor Elk-1 by ERK enhances ternary complex formation (TCF) between Elk-1, serum response factor (SRF) and the serum response element (SRE) present in several promoter sequences (Gille *et al.*, 1992; Marais *et al.*, 1993). Activation of TCF stimulates transcription of SRE containing genes including the transcription factor *c-fos* (Gille *et al.*, 1992; Janknecht *et al.*, 1993). Other nuclear targets include c-Myc, Ets-1 and ATF-2 (Abdel-Hafiz *et al.*, 1992; Seth *et al.*, 1992). $p90^{RSK}$, a cytoplasmic target of ERK phosphorylation (Sturgill *et al.*, 1988), also translocates into the nucleus where it can phosphorylate c-Fos and modulate transcription (Chen *et al.*, 1992; Chen *et al.*, 1993). The growth factor-induced stimulation of transcription is essential for cell proliferation and passage of cells through the G1 phase of the cell cycle. Induction of cyclin D1 expression and degradation of the cell cycle inhibitor $p27^{Kip1}$ induced by Ras-ERK signalling facilitates passage through G1 and entry into the S-phase of the cell cycle (Aktas *et al.*, 1997; Kerkhoff and Rapp, 1998; Lavoie *et al.*, 1996; Malumbres, 1998). Excessive Ras-ERK signalling can induce the expression of the inhibitor $p21^{Cip1}$ leading to growth arrest (Pumiglia and Decker, 1997; Tombes *et al.*, 1998) and may be a mechanism to regulate growth factor-induced cell proliferation (Malumbres, 1998).

Not all of the activated ERK translocates into the nucleus, with ERK phosphorylating several cytoplasmic proteins. ERK can stimulate arachidonic acid production by phosphorylation of cytoplasmic phospholipase A2 (cPLA2) (Kramer and Sharp, 1997; Lin *et al.*, 1993; Nemenoff *et al.*, 1993). Arachidonic acid is an important bioactive molecule required in the generation of prostaglandin, thromboxanes and leukotrienes (Needleman *et al.*, 1986). Myosin light chain kinase (MLCK) is also activated by ERK phosphorylation (Morrison *et al.*, 1996). Myosin activity, stimulated by myosin light chain phosphorylation, causes actin-myosin contraction and the generation of force within the cell (Kamm and Stull, 2001). This force

generation has been proposed to be required during integrin clustering at forming focal adhesions (Burridge *et al.*, 1997). In this regard, the stimulation of MLCK activity can target active ERK to newly forming focal adhesions where it may be involved in focal adhesion assembly or signalling (Fincham *et al.*, 2000). Klemke *et al.* (1997) have reported that inhibition of MLCK activity can suppress ERK1/2-induced cell migration on ECM (Klemke *et al.*, 1997). The activation of MLCK may therefore be an important effector in modulating cell adhesion and migration in response to ERK signalling. ERK can also phosphorylate SOS (Dong *et al.*, 1996); which may therefore allow feedback regulation of the Ras-ERK signalling cascade.

In recent years there have been an increasing number of reports suggesting MEK/ERK-independent effects by activated Raf. Mice in which the *raf-1* gene is knocked-out die during embryogenesis, yet cells derived from the embryo display normal ERK1/2 activation. This suggests that while *raf-1* is essential during development, Raf-1 may not be the *in vivo* MEK activator, which is likely to be B-Raf (Huser *et al.*, 2001). Phosphorylation of Raf -$Tyr^{340/341}$ is essential for activation of MEK kinase activity and substitution to phenylalanine residues prevents activation (Marais *et al.*, 1995). Mice in which *raf-1*$^{FF}$ was substituted for endogenous *raf-1*, apparently have a normal phenotype, even though Raf-$1^{FF}$ has no activity towards MEK. These viable mice show that Raf-1 function other than MEK activation is essential for development and survival (Huser *et al.*, 2001). Other biological processes independent of MEK/ERK activity are further described in Chapter 5 together with possible Raf effectors, in addition to MEK.

## 1.13 MAPK Signalling Cascade

This signalling cascade mediated by sequential phosphorylation and activation of proteins has parallels both in mammalian signalling systems and in model organisms. The Ras-ERK pathway in humans mediates mitogenic signalling. Other pathways regulate responses to cytokines and cell stress. In yeast, these signalling cascades regulate the mating response, cell-wall biosynthesis and glycerol accumulation

(Blumer and Johnson, 1994). Nature appears to have conserved this mechanism of sequential phosphorylation reactions to regulate a diverse array of cellular functions.

ERK1/2, activated by Ras, have several homologues in humans that are collectively known as the "MAP kinase superfamily" of proteins. This superfamily of proteins can be classified into subgroups comprised of ERKs, c-jun N-terminal kinases (JNKs) and p38 MAP kinases. These MAP kinases are components of several protein signalling cascades that are activated in response to a diverse array of stimuli. Figure 1.12 highlights the main components of these signalling pathways.

### 1.13.1 ERKs

ERK1/2 signalling stimulated by growth factors mediates mitogenic signalling. A total of seven ERKs have been identified in humans. After ERK1/2, ERK5 is the next best described member of this family. ERK5 is stimulated by growth factors (Kato *et al.*, 1997) and several components of the signalling pathway have been identified. ERK5 can synergise with ERK2 signalling (Pearson *et al.*, 2001) and this is described further in Chapter 5. Very little is currently known about the physiological role played by the remaining ERK proteins.

### 1.13.2 JNKs and p38 MAP Kinases

The c-Jun N terminal kinases (also known as the stress-activated kinases) are activated in response to cellular stress such as osmotic shock and UV irradiation; they are also activated by cytokines such as tumour necrosis factor α (TNFα) and interleukin-1 (IL-1) (Paul *et al.*, 1997; Weitzman, 2000). Stimulation of these pathways leads to cell apoptosis and growth arrest, but there is evidence to suggest that JNK signalling may modulate other cell – and - stimuli specific biological roles. JNKs are activated by phosphorylation of their consensus Thr-Pro-Tyr (TPY) motif, analogous to the TEY motif in the ERKs (Dhanasekaran and Premkumar, 1998). JNK phosphorylates several substrates including transcription factors, c-Jun, activated transcription factor 2 (ATF2), Elk-1 and the death domain protein MADD (Harper and LoGrasso, 2001)

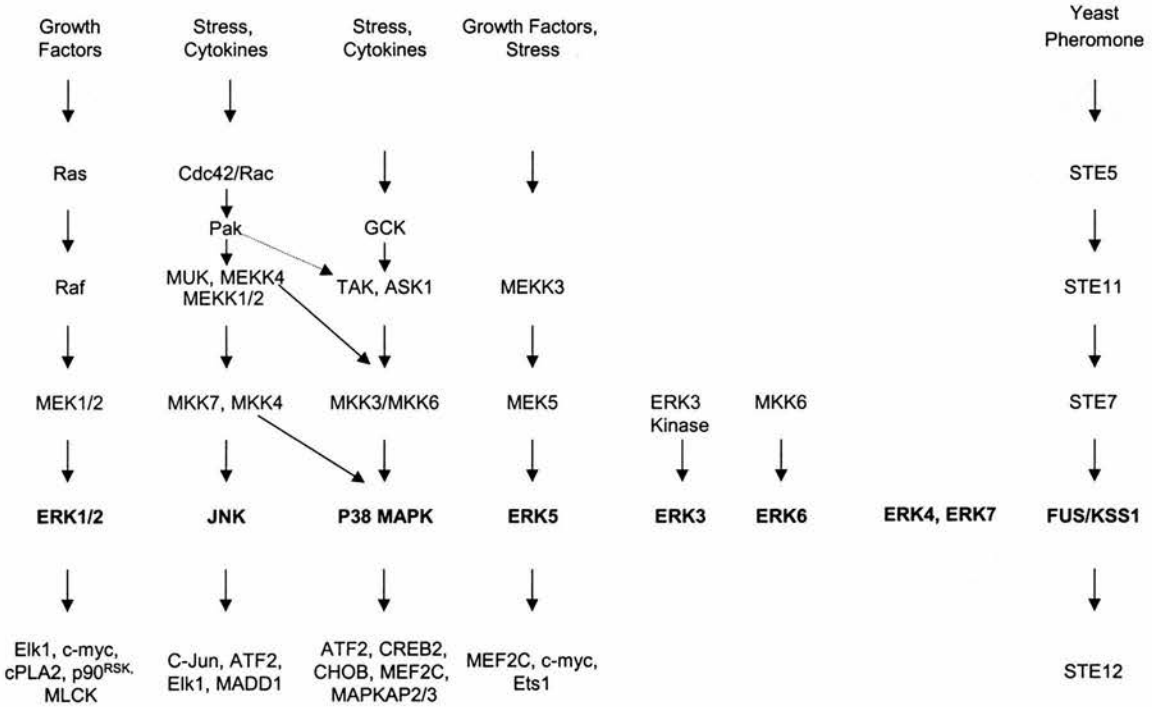


**Figure 1.12 MAPK Superfamily.**

Members of the MAPK superfamily (bold) are shown with their known upstream kinases and activating stimuli. Downstream targets of MAPK phosphorylation are also shown.

p38 MAP kinase, another stress activated protein kinase, is analogous to the high osmolarity glycerol responsive 1 (HOG1) pathway in yeast and is stimulated in response to growth factors, cellular stress and inflammatory cytokines. Phosphorylation of the Thr-Glu-Tyr (TGY) phosphorylation motif by MAPKK stimulates p38 MAP kinase activity (Dhanasekaran and Premkumar, 1998). p38 MAP kinase activity stimulates several transcription factors, activated transcription factor-2, c-AMP-responsive element, CHOP, myocyte enhance factor 2C and the kinase MAPKAPK-2. Activation of MAPKAPK-2 stimulates several transcription factors itself (Harper and LoGrasso, 2001). The response of p38 MAP kinase activity can lead to apoptosis, changes in the cell cycle and the upregulation of inflammatory cytokines during tissue inflammation (Ono and Han, 2000). Recent work has also shown that p38 MAP kinase is also required during development and differentiation (Nebreda and Porras, 2000).

# Integrins and Ras

Modulation of surface expression of integrins allows a cell to regulate the adhesive strength of a cell to ECM or counter receptors. Leucocytes can increase $\beta_2$ expression to facilitate recruitment to sites of tissue injury (Hillis and MacLeod, 1996). Cells can also change the integrin profile of a cell, thereby allowing cell migration to more suitable environments. Integrin profile changes have commonly been observed in tumour cells and have been described previously (reviewed in Varner 1996).

Ras is the most commonly mutated gene in human tumours (Bos, 1989). As previously described, Ras signalling can activate several pathways that can lead to transcriptional changes, which include changes in integrin expression levels. Expression of constitutively active Ras increased LFA-1 ($\alpha_L\beta_2$) expression in a thymocyte cell line (O'Rourke *et al.*, 1998) and $\beta_3$ expression in human melanoma and pancreatic carcinoma cell lines (Woods *et al.*, 2001). Conversely, Ras61L expression in an endothelial cell line displayed a reduction in $\alpha_3\beta_1$ integrin expression (Shin *et al.*, 1999). Reduction of oncogenic K-Ras expression in human colon carcinoma cells by antisense DNA increased $\alpha_1\beta_1$ and $\alpha_5\beta_1$ expression, while decreasing $\alpha_3\beta_1$ and $\alpha_V\beta_5$ expression (Schramm *et al.*, 2000). Expression of Ras G12V in a pro-B cell line, however, had no significant effect on $\alpha_4\beta_1$, $\alpha_5\beta_1$, LFA-1 and $\alpha_6\beta_1$ levels (Fujimoto *et al.*, 2001; Shibayama *et al.*, 1999; Tanaka *et al.*, 1999). Integrin expression levels in CHO cells expressing either $\alpha_{IIb}\beta_3$ or $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ ($\alpha\beta$-py cells) were also unaffected by expression of H-Ras G12V or R-Ras G38V (active) (Hughes *et al.*, 1997; Sethi *et al.*, 1999; Zhang *et al.*, 1996a). These results indicate that modulation of integrin surface expression levels by *ras* genes are both integrin - and - cell specific.

## 1.14 Signalling Pathways that Regulate Integrin Affinity

Integrin "activation", as previously discussed, results in the increase of ligand binding strength by the integrin. Activation is often measured by an increase in cell adhesion to immobilised substrates such as fibronectin, laminin and ICAM1. Adhesion is a complex process that requires a combination of integrin affinity and avidity changes. Signalling pathways that modulate cell adhesion therefore should not be solely ascribed to integrin affinity changes (Hughes P. and Pfaff, 1998). In this regard, cell binding to soluble ligands and ligand-mimetic antibodies are a better measure of affinity changes.

Soluble fibrinogen or PAC1 (the ligand-mimetic antibody of $\alpha_{IIb}\beta_3$) binding to platelets increases upon platelet activation by ADP, thrombin and epinephrine (Bennett and Vilaire, 1979). Agonist-induced activation of phospholipase $C_\beta$ by G-protein coupled receptors stimulates production of $IP_3$ and diacylglycerol (DAG) (Smith *et al.*, 1986b). $IP_3$-mediated release of intracellular $Ca^{2+}$ and DAG activation of protein kinase C induce activation of $\alpha_{IIb}\beta_3$ (Shattil *et al.*, 1998; Shattil and Brass, 1987). Platelets from mice deficient in $G\alpha_q$ fail to activate $PLC_\beta$ in response to agonists and are unable to aggregate (Offermanns *et al.*, 1997). Inhibition of agonist-induced tyrosine phosphorylation has also been shown to inhibit $\alpha_{IIb}\beta_3$ activation (Shattil *et al.*, 1992). Activation of $\alpha_{IIb}\beta_3$ by thrombin was inhibited by wortmannin treatment of platelets (Zhang *et al.*, 1996b). This suggests that activation of PI3-kinase and production of phosphoinositides may also be important for integrin activation.

ADP stimulation of $G\alpha_i$ and the inhibition of adenylyl cyclase is also important for platelet aggregation. ADP-dependent platelet aggregation in mice lacking $G\alpha_{i2}$ is strongly inhibited due to a failure in the activation of $\alpha_{IIb}\beta_3$ (Jantzen *et al.*, 2001). $\alpha_{IIb}\beta_3$ expressed in human B-lymphocytes is activated in response to formyl Met-Leu-Phe (fMLP) treatment. Activation was sensitive to pertussis toxin treatment and a PKC inhibitor, suggesting that $G_{\alpha i}$ and PKC were required for integrin activation (Qi *et al.*, 1998).

$\alpha_{IIb}\beta_3$ expressed in CHO cells, however, failed to respond to any $\alpha_{IIb}\beta_3$-stimulating agonists including ADP, thrombin and phorbol esters (O'Toole *et al.*, 1990). $\alpha_{IIb}\beta_3$ on CHO cells can be activated by stimulation of the co-expressed platelet glycoprotein Ib-IX (GPIb-IX) (Gu *et al.*, 1999) in a pathway dependent on protein kinase G (PKG) activation of Raf-MEK-ERK1/2 (Li *et al.*, 2001). However, independent activation of the PKG-ERK pathway in the absence of GPIb-IX activation failed to reproduce $\alpha_{IIb}\beta_3$ activation.

### 1.14.1 Small GTP-binding proteins, H-Ras and R-Ras

Substitution of the $\alpha_{IIb}\beta_3$ cytoplasmic domain with that of the fibronectin ($\alpha_5\beta_1$) or laminin ($\alpha_{6A}\beta_1$) integrin produces a constitutively active chimeric integrin in CHO cells (O'Toole *et al.*, 1991; O'Toole *et al.*, 1994). Expression of Ras G12V in these cells suppressed this active integrin conformation in a pathway sensitive to MAP kinase phosphatase1 expression (Hughes *et al.*, 1997). In contrast Ras Q61L expression in a haematopoietic cell line can activate the native $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrin in an ERK1/2 independent manner (Liu *et al.*, 1999; Shibayama *et al.*, 1999). In a mast cell line, $\alpha_5\beta_1$ activation by Ras G12V was sensitive to the PI3-kinase inhibitor, wortmannin (Kinashi *et al.*, 2000). Integrin affinity modulation by active Ras may therefore be integrin, cell and integrin affinity-state specific.

Expression of active R-Ras G38V in CHO cells can activate $\alpha_{IIb}\beta_3$ and reverse Ras G12V suppression of the chimeric integrin (Sethi *et al.*, 1999; Zhang *et al.*, 1996a). Effectors of R-Ras integrin affinity modulation remain to be identified, although C-Terminal prenylation (Oertli *et al.*, 2000) and a putative SH3 binding domain within R-Ras have been shown to be important (Wang *et al.*, 2000). A small death-domain containing protein, PEA-15, can reverse Ras G12V integrin suppression in a pathway impaired by dominant negative R-Ras (Ramos *et al.*, 1998). Activation of Ephrin receptors, a receptor tyrosine kinase, led to R-Ras phosphorylation and decreased adhesion to ECM proteins. It is proposed that disruption of R-Ras effector binding through phosphorylation of $Tyr^{66}$ within the effector domain may also impair R-Ras signalling and therefore facilitate the loss in ECM adhesive strength (Zou *et al.*,

1999). The recruitment of the protein tyrosine phosphatase, SHP2 to the receptor and dephosphorylation of FAK and paxillin may contribute to the loss in adhesion (Miao *et al.*, 2000). By contrast, activation of Ephrin-ligand expressing cells by their receptors display an increase in adhesion to ECM in a $\beta_1$ dependent manner (Davy and Robbins, 2000; Huai and Drescher, 2001). Affinity of the $\beta_1$ integrins remains to be determined, though it is interesting to note that activation of receptor and ligand can have opposite effects on integrin-mediated cell adhesion.

### 1.14.2 Other Signalling Molecules

Inhibition of PI3-kinase activity can inhibit activation of $\alpha_{IIb}\beta_3$ in platelets (Zhang *et al.*, 1996b). The production of 3-phosphorylated phosphoinositides may allow the recruitment of proteins containing PH domains to the plasma membrane. Cytohesin, a pleckstrin homology (PH) domain containing protein is recruited to the plasma membrane upon PI3-kinase activation (Nagel *et al.*, 1998). Its interaction with the cytoplasmic tail of LFA-1 can induce adhesion to ICAM-1 (Kolanus *et al.*, 1996) and can also generate an activation epitope on LFA-1 recognised by the antibody mAb24 (Dransfield *et al.*, 1992; Geiger *et al.*, 2000).

Rap1, another small GTP binding protein has also been shown to induce activation dependent epitopes in LFA-1. In a pro-B-cell line, crosslinking of the T cell receptor induced activation of LFA-1 that was impaired by expression of a dominant negative Rap1 construct (Katagiri *et al.*, 2000). In Jurkat cells, crosslinking of CD31 (PECAM-1) induced mAb24 binding to LFA-1 through the activation of Rap-1 (Reedquist *et al.*, 2000). Rap-1 can activate B-Raf (Ohtsuka *et al.*, 1996) although no activation of ERK1/2 was observed with CD31 crosslinking.

In lymphocytes, the chemokine stromal derived factor-1 (SDF-1) can induce soluble ICAM-1 binding to LFA-1 in a process that is inhibited by pertussis toxin (Constantin *et al.*, 2000) whereas upregulation of $\alpha_4\beta_1$ activity required the Src kinase $p56^{Lck}$ that was potentiated by the phosphatase inhibitor pervanadate (Feigelson *et al.*, 2001).

Isolated $\beta_1$ cytoplasmic tails can suppress the chimeric integrin in CHO cells (Chen *et al.*, 1994). CD98, an early T-cell activation antigen, can associate with $\beta_1$ integrins and reverse this integrin suppression (Fenczik *et al.*, 1997; Fenczik *et al.*, 2001). Crosslinking of CD98 can stimulate adhesion to ECM (Fenczik *et al.*, 1997) and PI3-kinase activity (R. Rintoul, personal communication). Whether PI3-kinase activity is required for reversal of integrin suppression remains to be determined.

## 1.15 Aims of this Thesis

The mechanisms modulating integrin affinity are currently poorly understood. Active H-Ras (Ras G12V) has been shown to suppress the active chimeric integrin ($\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$) expressed in CHO cells ($\alpha\beta$-py cells) in a pathway sensitive to MKP-1 expression (Hughes *et al.*, 1997).

The aims of this thesis were:

1. Establish the $\alpha\beta$-py integrin affinity reporter and transfection system.
2. Reproduce the initial findings of integrin suppression by Ras G12V.
3. Examine the requirement of ERK1/2 activation for integrin suppression.
4. Determine which Ras effector pathways mediate integrin suppression.
5. Identify new components of the integrin suppression pathway.

# MATERIALS AND METHODS: CHAPTER 2

## 2.1 Materials

All chemicals were purchased from Sigma (Dorset, U.K.) unless otherwise stated. The following reagents were obtained from Life Technologies (Paisley, UK): Dulbecco's Modified Eagles Medium (DMEM); Penicillin; Streptomycin; dialysed foetal bovine serum (FBS); L-glutamine; 30% (w/v).

Antibodies were obtained from the following sources: anti-Ha (Y-11), anti-myc (9E10 and A14), anti-ERK2 (C-14), anti-Raf (E-10) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Phospho-ERK1/2 (ERK-PT115) was from Sigma (Dorset, UK) and Phospho-Akt (Ser 473) was from New England BioLabs (Beverly, USA). Anti-PKBα antibody was a kind gift from Dr. D. Alessi (Department of Biochemistry, University of Dundee). Anti-RalA antibody was from Transduction Laboratories (Lexington, USA). All anti-species specific horseradish peroxidase-conjugated antibodies were from DAKO (Bucks, UK).

DNA constructs Tac-α5 (permission from Dr. S. E. LaFlamme, Centre for Cell Biology and Cancer research, NY, USA), pDCR-H-Ras (G12V and effector mutants) (permission from Dr. M. H. Wigler, Cold Spring Harbour Laboratory, NY, USA), pSG5-p110-CAAX and pSG5-R-Ras G38V (permission from Prof. J. Downward, ICRF, London, UK) were all provided by Dr. M. H. Ginsberg (Scripps Research Institute, La Jolla, USA) and have been described previously (LaFlamme *et al.*, 1992; Oertli *et al.*, 2000; Wennstrom and Downward, 1999; White *et al.*, 1995). Dr. S. M. Keyse (ICRF, Dundee, UK) provided pSG5-MKP-1 (Keyse and Emslie, 1992). pCDNA3-Raf-BxB CAAX was provided by Dr. C. K. Weber (University of Ulm, Germany) (Daub *et al.*, 1998). pEFm-A-Raf, B-Raf and Raf-1 were provided by Dr. R. Marais (Institute of Cancer Research, London, UK) (Marais *et al.*, 1997). Dr. J. L. Bos (University of Utrecht, Utrecht, Netherlands) provided pMT2-RalA (WT, 23V and 28N) (Wolthuis *et al.*, 1997) and pGEX-RalBD (Wolthuis *et al.*, 1998). Dr. M. White (University of Texas Southwestern Medical Centre, Texas, USA) provided

pCDNA3-Raf-BxB T481A (Pearson *et al.*, 2000). Dr. M. H. Cobb (University of Texas Southwestern Medical Centre, Texas, USA) provided pCMV5-ERK2-MEK1 (WT and KD) (Robinson *et al.*, 1998). pCMV-Ha-MEK1DD (permission from Dr. M. J. Weber, University of Virginia, Charlottsville, USA) was provided by Prof. W. Kolch (Beatson Institute for Cancer Research, Glasgow, UK) (Catling *et al.*, 1995). Drosophilia DNA pWRhpA-Auk and pBluescript-Cass were provided by Dr. N. Brown (Cambridge University, Cambridge, UK). The small cell carcinoma cDNA library (H33) was obtained from the UK Human Genome Mapping Project Resource Centre, Cambridge, UK.

## 2.2 Cell Culture

The αβ-py CHO cell line was a kind gift from Dr Mark Ginsberg (Scripps Institute, La Jolla, USA). This cell line stably expresses a chimeric integrin comprised of $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ (Hughes *et al.*, 1997). Expression of this integrin is maintained through G418 selection and expression driven by the polyoma T antigen.

The αβ-py cell line was cultured in Dulbeccos Modified Eagles Media (DMEM) (Sigma), supplemented with 10% (v/v) foetal bovine serum (FBS) (heat inactivated at 57°C for 1 hour), 1% (v/v) non-essential amino acids (Life Technologies) and G418 antibiotic (400μg/ml) (Promega) to maintain expression of the integrin.

Quiescent media for αβ-py cells was supplemented with 0.1% (v/v) heat inactivated FBS rather than 10% (v/v).

All media contained 50 IU/ml penicillin, 50 IU/ml streptomycin and 5mg/ml L-glutamine and all cell lines were maintained in a humified atmosphere of 5% CO2/95% air at 37°C. All cell lines were used at the lowest possible passage number and regularly screened for mycoplasma infection.

## 2.3 Chemical competence and transformation of E.Coli

### 2.3.1 Generation of chemical competent E.Coli

Transformation competent E. Coli strains DH5α and BL21 were purchased from Life Technologies (Paisley, UK), strains MC0161/P3, TOP10 and TOP10/P3 were purchased from Invitrogen (Groningen, Netherlands). Additional competent stocks of DH5a, BL21 and TOP10 were made according to the method by (Hanahan, 1983). An overnight culture of E.Coli (0.8-1% v/v) was used to inoculate 250 ml of SOC media (Life Technologies, Paisley, UK) and grown (18°C at 225rpm) to an absorbance at 600nm of 0.4-0.8 units. All subsequent steps to generate competent cells were performed on ice. Cells were pelleted (3000 rpm at 4°C) in a Sorvall RT6000B centrifuge (Stevanage, UK) and gently resuspended in 84 ml chilled transformation buffer containing 10mM PIPES, 15mM $CaCl_2.2H_2O$, 25mM KCl, 55mM $MnCl_2.4H_2O$, pH6.7. Following a 10-minute incubation on ice, cells were pelleted and resuspended in 20 ml transformation buffer. Dimethyl sulphoxide (7.5% v/v) was added and cells incubated for a further 10-minutes on ice. Competent cells were aliquoted into chilled eppendorfs and snap frozen in liquid nitrogen. All competent cells were stored at -70°C.

### 2.3.2 Transformation of E.Coli

Frozen aliquots of cells were thawed on ice and transferred to chilled eppendorfs. In the case of MC0161/P3 and TOP10/P3 cells, β-mercaptoethanol (25mM final concentration) was added to cells prior to addition of DNA. DNA (1-10ng) was gently mixed with the cells and allowed to rest for 30 minutes on ice. Cells were heat-shocked at 42°C in a thermomixer (Eppendorf, Cambridge, UK) for 45-60 seconds and returned onto ice for a further 2 minutes. Transformed cells were allowed to grow by incubating the cells in antibiotic free SOC media for 60 minutes at 37°C (225 rpm in thermomixer), prior to spreading on appropriate antibiotic selection plates. Plates were incubated at 37°C overnight in an incubator (Stuart

Scientific, UK). Individual colonies were picked into LB broth containing antibiotics and grown overnight for DNA purification and restriction enzyme digest verification.

## 2.4 DNA Purification

Small scale (1-10ml) DNA purifications were performed using Wizard SV Miniprep kits (Promega) as per manufacturers instructions. Diagnostic restriction digests were performed with appropriate restriction enzymes (Promega) and resolved on 1% agarose (Seakem, Rockland, Maine, USA) gels containing 0.3μg/ml ethidium, bromide to enable UV visualisation.

Large scale (100-500ml) DNA purifications were performed using the Qiagen Endotoxin Free Maxiprep kit (Qiagen, Crawley, UK) as per manufactures instructions. For low copy plasmids, the Qiagen Midiprep kits were used together with their low copy number plasmid protocol. Purified DNA was checked with diagnostic digests and quantified using on the Pharmacia Biotech Ultrospec 2000 UV spectrophotometer (Amersham Pharmacia Biotech, Amersham, U.K)

## 2.5 Cell Transfection

Transient transfections of αβ-py and CHO-K1 cells were performed with Lipofectamine™ Plus reagent (Life Technologies) as described by Chen *et al.* (1994) (Chen *et al.*, 1994). Cells were seeded at an appropriate density to reach 50-70% confluency overnight in 100/60mm dishes (Corning, High Wycombe, UK). Purified DNA was placed in 5ml BD Falcon tubes (Part No: 352034, Oxford, UK) in a volume ranging from 1-20μl, the DNA was pre-complexed with 110μl of the Plus reagent mixture and gently mixed. The Plus reagent mixture contained Plus reagent 0.25% (v/v) diluted in DMEM containing 1% (v/v) non-essential amino acids DMEM (+AA). Following a 15 min incubation at room temperature, the DNA was complexed with 110μl of the Lipofectamine mixture and gently mixed. The Lipofectamine reagent contained Lipofectamine 0.25% (v/v) diluted in DMEM (+AA). After a further 15 min incubation at room temperature, the transfection

mixture was made up to a final volume of 4 ml with pre-warmed DMEM (+AA) and placed onto washed cells. Cells were cultured for 5 hrs at 37°C and then fed with complete media (4 ml). Twenty four hours after transfection, media containing DNA and Lipofectamine was removed and replaced with fresh complete media. For experiments when protein kinase activities were to be assessed, the transfection media was replaced with quiescent media. For PD098059 (Calbiochem, Nottingham, UK) studies, the inhibitor was added to quiescent media at the appropriate concentration and time as stated. Forty eight hours post transfection; cells were either lysed for SDS-PAGE anlaysis or used for integrin affinity determination.

All plasmids used for transient transfections were titrated for maximal expression in the αβ-py cells. Subsequent studies experiments for integrin affinity determination or western blot analysis were performed on transfected cells using optimal DNA quantities unless otherwise stated.

## 2.6 Assessment of protein concentration

Protein concentrations were quantified using a BCA protein assay (Pierce, IL, USA). A protein standard curve was generated using bovine serum albumin (Sigma) at a range of 0.1-0.5mg/ml diluted in 1:10 lysis buffer. Samples were diluted 1 in 10 in $dH_2O$ and 10μl incubated with 200μl BCA reagent (30 minutes, 37°C) in 96 well plates. Protein concentration was determined using an automated plate reader (MRX microplate reader, Dynatech, Chantilly VA).

## 2.7 SDS Page and Western Blotting

### 2.7.1 Cell Lysis

Transfected cells were washed once with ice cold PBS and lysed in buffer containing 50mM Hepes (Na Salt), 0.3M NaCl, 1.5mM $MgCl_2$, 1.2mM EDTA, 0.5% Triton X-100, 20mM β-glycerophosphate, 100mM sodium fluoride, 10mM sodium pyrophosphate, 1mM sodium vanadate, 0.5mM dithiothreitol (DTT). One Complete™ protease inhibitor tablet (Boehringer Manheim, Lewes, UK) was added

per 50ml of lysis buffer. Lysates were cleared by centrifugation at 13000 rpm for 10 minutes at 4°C, assessed for protein concentration (described in 2.6) and solubilised in 4 x sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer at 99°C for 5 minutes.

### 2.7.2 SDS PAGE and Western Immunoblotting

Samples were cooled and equal amounts of protein (10-100 μg) were resolved on SDS polyacrylamide gels using a vertical electrophoresis tank Biorad Protean 3 system (Biorad, Hemel Hempstead, UK) and (ATTO Corp., Japan). Samples were electrophoresed at 100-150 Volts using SDS-Tris-Glycine electrophoresis buffer for 1-2 hours beside pre-stained molecular weight markers (Life Technologies, Paisley, U.K.). For optimal separation, 8% gels were used for 60-120 kDa proteins, 10% gels for 40-70 kDa proteins and 12% gels for 10-40 kDa proteins.

Proteins were transferred onto Hybond C nitrocellulose membranes (Amersham Pharmacia Biotech, Amersham, U.K.) in a methanol based transfer buffer at 400 milliamps for 1 hour using a Mini Protean II blotting tank (Biorad, UK). Equal protein loading was confirmed by incubating blots for 5 minutes in Ponceau S (Sigma), which allows visualisation of protein bands. Non specific protein binding sites on the membranes were blocked by incubation in PBS-Tween 20 containing 5% non-fat dried milk powder for 1 hour at room temperature. Membranes were probed with appropriate primary antibodies (Table 2.1) diluted in PBS-Tween 20 containing 5% non-fat dried milk powder overnight at 4°C. Membranes were washed in PBS-Tween 20 (3 x 30 second vigorous washes and 3 x 10 minute washes) before species appropriate horseradish peroxidase (HRP) conjugated secondary antibody (DAKO, Ely, UK) (diluted in washing buffer) was added for 1 hour (Table 2.1). Finally, membranes were rewashed as above and immunoreactive bands were identified using enhanced chemiluminescence (ECL) (Amersham Pharmacia Biotech, Amersham, U.K) as per manufacturer's instructions.

**Table 2.1 Immunoblotting antibody dilutions**

| Primary Antibody | Dilution | Secondary Antibody | Dilution |
|---|---|---|---|
| Ha Antibody (Y-11) | 1:500 | Anti-Rabbit HRP | 1:1000 |
| Myc Antibody (9E10) | 1:1000 | Anti-Mouse HRP | 1:1000 |
| Myc Antibody (A14) | 1:1000 | Anti-Rabbit HRP | 1:1000 |
| Phospho-ERK1/2 | 1:2000 | Anti-Mouse HRP | 1:1000 |
| ERK2 (C-14) | 1:2000 | Anti-Rabbit HRP | 1:1000 |
| Phospho-Akt | 1:500 | Anti-Rabbit HRP | 1:1000 |
| Akt-alpha | 1:500 | Anti-Sheep HRP | 1:1000 |
| RalA | 1:1000 | Anti-Mouse HRP | 1:1000 |
| Raf (E-10) | 1:1000 | Anti-Mouse HRP | 1:1000 |

*4 x SDS-PAGE sample buffer*: 50mM Tris-HCl, 10% (v/v) glycerol, 2% (v/v) SDS, 0.1% (v/v) bromophenol blue and 10% (v/v) β-mercapoethanol.

*SDS polyacrylamide gels*: Separating Gel – 0.375M Tris base (pH8.8), 0.1% (v/v) SDS, 8-12% (v/v) acrylamide (Life Technologies, Paisley, UK), 0.1% (v/v) ammonium persulphate and 0.08% (v/v) TEMED

Stacking Gel – 0.13M Tris base (pH8.8), 0.15% (v/v) SDS, 4.6% (v/v) acrylamide, 0.13% (v/v) ammonium persulphate and 0.1% (v/v) TEMED

*Gel electrophoresis buffer*: 5 mM Tris base (pH 8.3), 25 mM glycine and 0.01% (v/v) SDS.

*Transfer buffer*: 210 mM glycine, 24.7 mM Tris base and 20% (v/v) methanol.

*PBS-Tween 20 Wash Buffer*: PBS containing 0.2% (v/v) Tween 20 (Sigma)

## 2.8 Integrin Affinity Determination by Flow Cytometry

### 2.8.1 Cell Staining

Integrin affinity in transfected cells was analysed by three colour flow cytometry forty eight hours post transfection. Cells were transfected with test DNA (0-10μg) together with 0.75μg Tac-$\alpha_5$ transfection reporter construct (extracellular domain of

the IL-2 receptor and the intracellular domain of the $\alpha_5$ integrin). Single cell suspensions of trypsinised cells were resuspended in a total volume of 50µl containing 0.4% (v/v) PAC1 ascites (gift from S. Shattil, Scripps Institute, La Jolla, USA) (Shattil *et al.*, 1985) for 30 minutes at room temperature in Hepes/NaCl buffer (20mM Hepes, 140mM NaCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 2mg/ml Glucose, pH 7.4). Internal controls for each sample were performed containing either 5mM EDTA or 100µM $MnCl_2$ or 2µM Ab33 (activating antibody). Cells were washed with cold PBS and incubated on ice with 50µl DMEM containing 4% (v/v) anti-mouse IgM-FITC (Biosource, Nivelles, Belgium) for 30 minutes in the dark. Cells were washed again and incubated on ice for a further 30 minutes with 50µl DMEM containing 2% (v/v) anti-Tac-R-phycoerythrin (R-PE) (DAKO, Ely, UK). Cells were finally washed and resuspended in cold PBS. Immediately prior to analysis on a FACS-Caliber (BD, Erembodegem, Belgium), ToPro3 (Molecular Probes, Leiden, Netherland) at a final concentration of 1µM (in PBS) was added to each sample.

### 2.8.2 FACS and data analysis

PAC1 binding was determined by gating for live and highly transfected cells (ToPro3 negative and high Tac binding respectively). To obtain numerical estimates of integrin activation, an integrin activation index (AI) was calculated.

$$AI = \left(\frac{F_N - F_I}{F_A - F_I}\right) \times 100 \text{ and } Percent\ Inhibition = \left(\frac{AI_O - AI}{AI_O}\right) \times 100$$

$F_N$ : Geometric mean fluorescence intensity (MFI) of PAC1 binding of the native integrin

$F_I$ : MFI of PAC1 binding in the presence of 5mM EDTA

$F_A$ : MFI of PAC1 binding in the presence of 100µM $Mn^{2+}$ or 2µM Ab33

$AI_O$ : Activation index with the control vector

AI : Activation index with DNA under test

## 2.9 Gene Subcloning

Drosophila genes cassowary and auk were obtained from Dr. N. Brown (Cambridge, UK). Cassowary was cloned into the DNA cloning vector pBluescriptSK and auk into the pWRhpA *drosophila* transformation vector, neither were mammalian expression vectors.

The Ral genes were obtained from J.Bos (Utrecht, Netherlands) and were cloned into the pMT2 vector that expresses poorly in αβ-py CHO-K1 cells.

### 2.9.1 High fidelity PCR of genes

Primers were designed flanking the gene of interest with each forward primer and reverse primers containing appropriate restriction sites (Table 2.2). All primers were screened against Genbank databases to check for mismatches or cross-reactivity's, none were found. High fidelity PCR was performed using Pfu Turbo DNA polymerase (Stratagene, Amsterdam, Netherlands). Twenty nanograms of template DNA were amplified in a reaction volume of 50μl containing (1X Pfu reaction buffer (contains 2mM $MgSO_4$), 0.2mM dNTPs each, 0.2μM of each primer and 2.5 U of Pfu polymerase). Reactions were performed on a MWG Primus 96 Plus machine (Ebersberg, Germany), with cycle conditions as described in (Table 2.3). PCR products were resolved on a 1% agarose gel containing 0.3μg/ml ethidium bromide to enable UV visualisation.

**Table 2.2 Gene cloning primers**

| Target cDNA | Primer Sequence (F=Forward, R=Reverse) |
|---|---|
| cass | F: CCGGGATCCATGGACAACATAGAGCGACTGTACAAATGC<br>R: CTAGAAGCTTTTAGTACTTGTTGAATCGCCTGTTTCTGTAGCCTC |
| auk | F: CCGGAATTCATGAGCAGTCCCAGGGATAAG<br>R: CTAGAAGCTTCTAATTTACGGGCGATATGGTCTG |
| ral | F: CCGGGATCCATGGCTGCAAATAAGCCCAAG<br>R: CTAGAAGCTTTTATAAAATGCAGCATCTTTCTCTG |

Underlined sequence highlights the incorporated restriction enzyme sequences.

**Table 2.3 PCR Cycle conditions for gene cloning**

| Cycle Stage | Cass PCR | Auk PCR | Ral PCR |
|---|---|---|---|
| Stage 1 | 94°C for 1 min | 94°C for 1min | 95°C for 1 min |
| Stage 2 | 94°C for 30 sec<br>70°C for 30 sec<br>72°C for 2 min<br>25 cycles | 96°C 30 sec<br>67°C for 4 min<br><br>30 cycles | 95°C for 1 min<br>62°C for 1 min<br>72°C for 1 min<br>25 cycles |
| Stage3 | 72°C for 10 min | 72°C for 10 min | 72°C for 10 min |

### 2.9.2 Cloning of genes into pGEMT Vector

PCR products were ethanol precipitated and 500ng of the product were used in a poly-A tail reaction containing 2mM dATP and 2.5U standard Taq polymerase (Promega, Southampton, UK), reactions were performed for 30 minutes at 70°C. Poly-A tailed PCR products were ligated into the pGEMT vector (Promega) using the Rapid ligation kit. Ligations were performed as per manufacturers instructions using 50ng of precut pGEMT vector and a 3:1 insert ratio. Following a 2 hour ligation at room temperature, 50% of the ligation mixture was transformed into DH5α E.Coli and spread onto LB agar plates containing 50μg/ml ampicillin, 0.5mM isopropy-beta-D-thiopyranoside (IPTG) and 80μg/ml 5-Bromo-4-chloro-3-indolyl beta-D-galactopyranoside (X-Gal). White colonies were subsequently amplified and screened for correct insert size by appropriate restriction digests, cass/ral (BamH1-HindIII) and auk (EcoR1-HindIII). Successful ligations were sent for sequencing at the CIR sequencing facility using the ABI automated fluorescence sequencing kit. Sequences from clones that matched template DNA were subsequently used for sub-cloning into the mammalian expression vector, pCMV-Tag3B (Stratagene; Amsterdam, Netherlands).

### 2.9.3 Sub-cloning genes into pCMV-Tag3B vector

Both insert containing plasmid and pCMV-Tag3B (Stratagene, Amsterdam, Netherlands) (5μg) (target plasmid) were digested with 20U of BamH1 and HindIII in a final volume of 30μl, for 2 hours at 37°C. Following digestion, pCMV-Tag3B

DNA was treated with 20U shrimp alkaline phosphatase (Boehringer Manheim, Lewes, UK) for 30 minutes at 37°C. Dephosphorylation of free terminal phosphate groups on the target plasmid was terminated by incubating the mixture at 65°C for 15 minutes. Ligation control samples included single cut pCMV-Tag3B (BamH1 alone) and unphosphatased vector. All digestions were gel purified using the Qiagen Gel extraction kit (Crawley, UK) as per manufacturers instructions. Purified DNA was quantified as described previously (2.4). Ligation into the target plasmid was performed using 100ng cut vector and a 3:1 molar ratio of insert with 3U T4 DNA ligase (Promega, Southampton, UK) in a final volume of 10μl. Ligations were performed using a cycling PCR reaction of 14°C for 30 seconds and 24°C for 30 seconds, for a total of 2 hours. Control ligations were performed in the absence of insert or with single cut vector. Following ligation, the ligation mixture was transformed into DH5α E.Coli as described previously (2.3.2). Positive colonies were amplified and screened with restriction digests.

## 2.10 Library Amplification

Mammalian cDNA expression library H33 (Small cell lung carcinoma) was obtained from the Human Genome Mapping Project (Cambridge, UK). The library was cloned into the pCDM8 vector (Invitrogen, Groningen, Netherlands) that contains a CMV promoter and the polyoma origin of replication (facilitates replication in αβ-py cells) with insert sizes greater than 500bp. Library DNA (15ng) was transformed into either MC0161/P3 or TOP10/P3 E.Coli strains (3x100μL) as described in 2.3.2. Transformations were subsequently spread onto 25x25cm (Corning, High Wycombe, UK) LB-Agar plates containing 50μg/ml ampicillin and 10μg/ml tetracycline and plates were incubated overnight at 37°C. Transformations were repeated six times to obtain sufficient number of colonies as to cover the quoted complexity of the library ten times.

Colonies were scraped from each plate into antibiotic free LB-Broth and DNA purified from the culture using the Qiagen Midiprep-Kit following the low copy

number plasmid protocol. Purified DNA was quantified by UV spectrophotometry and verified by restriction digestion with the XhoI restriction enzyme. DNA from individual preparations were pooled and used in the genetic screen.

## 2.11 Library Screening

### 2.11.1 Cell Sorting

The genetic screen of the SCLC cDNA expression library was performed as described in Fenczik *et al.* (1997). Transfection of αβ-py cells was performed as described previously (2.5), in 100mm tissue culture plates. A total of 19 plates were transfected: pCDM8 (4μg), Ras G12V (3μg) and 17 plates with H33 library DNA (4μg) all with 2μg of Tac-$\alpha_5$ reporter construct. Integrin affinity determination was performed 48 hours after transfection as described in 2.8. Sixteen library transfected plates were trypsinised and resuspended in a total of 1.35ml of Hepes/NaCl buffer, single cell suspension of cells (6 x 225μl) were incubated with 25μl PAC1 ascites fluid to give a final concentration of 0.4% (v/v). Cells were washed with cold PBS and incubated on ice with 250μl DMEM containing 4% (v/v) anti-mouse IgM-FITC (Biosource, Nivelles, Belgium) for 30 minutes in the dark. Cells were washed again and incubated on ice for a further 30 minutes with 250μl DMEM containing 2% (v/v) anti-Tac-PE (DAKO, Ely, UK). Cells were finally washed and resuspended in cold PBS at a concentration of 2 x $10^6$ cells/ml.

Cell sorting was performed on a FacsVantage cell sorter (BD, Erembodegem, Belgium) with a 70-micron flow nozzle. Immediately prior to cell sorting, 7-AAD (Molecular Probes, Leiden, Netherland) a cell viability dye at a final concentration of 20ng/ml was added to each of the six sort tubes. Cells transfected with pCDM8 and Ras G12V enabled the sort gate, 7-AAD negative (live), Tac positive (transfected) and low PAC1 binding (suppressed integrin) to be set (Figure 6.11). Cells collected in 3ml DMEM were subjected to HIRT extraction of plasmid DNA (Hirt, 1967).

Briefly, cells were transferred to 1.5ml eppendorfs and centrifuged at 1000 rpm for 10 minutes and resuspended in 400µl lysis solution (0.6% SDS (w/v) and 10mM EDTA). Following a 20 minute incubation at room temperature, 100µl of 5M NaCl solution was added and the mixture incubated at 4°C overnight. Lysates were clarified by centrifugation at 13000 rpm for 10 minutes and subjected to two cycles of phenol/chloroform purification (lysate: phenol/choloroform 1:1 ratio). DNA within the aqueous phase was subjected to ethanol precipitation with 20µg of glycogen (Boehringer Manheim, Lewes, UK). DNA resuspended in 100µl $dH_2O$ was precipitated with 3M sodium acetate 10% (v/v) and 300% (v/v) ethanol by centrifugation at 13000 rpm for 20 minutes. The DNA pellet, washed with 70% ethanol, was resuspended in a final volume of 8µl $dH_2O$.

DNA was digested overnight with 10 units Dpn1 restriction enzyme at 37°C and used to transform TOP10/P3 E.Coli (Invitrogen, Groningen, Netherlands) as described in 2.3.2. Individual bacterial colonies were grown, stored and pooled into groups of 10 for plasmid purification (midi-prep) as described in 2.4. Library pools (4µg) were transformed into αβ-py cells and re-screened for integrin affinity modulation by Facs analysis.

## 2.12 Ral Activity Assay

### 2.12.1 Production of GST-RalBD agarose beads

LB broth (500ml) containing 50µg/ml ampicillin was inoculated with BL21 E.coli transformed with the pGEX-GST-RalBD construct and grown (37°C at 225rpm) to an absorbance at 600nm of 0.4-0.6 units. IPTG was added to a final concentration of 0.2mM and the culture was allowed to grow for a further 3 hours. Bacteria were centrifuged at 4000 rpm for 25 minutes and resuspended in 10 ml lysis solution (PBS, 5mM DTT, 2mg/ml lysozyme (Sigma) and Complete™ protease inhibitor tablet). All subsequent steps were performed on ice. Following a 30 minute incubation on ice, bacteria were sonicated (Ultrawave U50, Belmont Instruments, Glasgow, UK) 4 times for 30 seconds on ice and then Triton X-100 added 2% (v/v).

Bacterial lysates were placed on a roller for 20 minutes at 4°C; lysates were then clarified by centrifugation at 12000 rpm for 30 minutes at 4°C. Bacterial lysates mixed with 1 ml expanded glutathione-agarose beads (Sigma, Poole, UK) were incubated on a roller overnight at 4°C. Beads were centrifuged at 1500 rpm for 15 minutes at 4°C and resuspended in 1 ml 100% glycerol and stored at -20°C until required.

### 2.12.2 Ral Assay

Cells transfected (100mm dishes) as described in 2.5 were quiesced 24 hours prior to lysis. Cells washed in ice-cold PBS were lysed in 450µl Ral lysis buffer (50mM Tris-HCl, pH7.4, 200mM NaCl, 2.5mM $MgCl_2$, 1% NP-40 (v/v), 15% glycerol (v/v) and Complete™ protease inhibitor tablet). Lysates were clarified by centrifugation at 13000 rpm for 10 minutes at 4°C and protein concentration estimated as described previously. Protein concentrations were normalised and 400µl of each cell lysate were incubated with 50µl of GST-RalBD-agarose beads for 60 minutes on a roller at 4°C, the remaining 50µl of cell lysate was solubilised with 4 x SDS-PAGE sample buffer at 99°C for 5 minutes. Agarose beads were washed 4 times with Ral lysis buffer and finally resuspended in 30µl 1 x SDS-PAGE sample buffer. Whole cell lysates and Ral activity assay samples (20µl) were analysed by SDS-PAGE on a 15% gel and western blots were probed with an anti-RalA monoclonal antibody (Transduction Laboratories, Oxford, UK).

## 2.13 NF-κB Binding Assay

### 2.13.1 Nuclear Protein Extraction

Cells were transfected as described in 2.5 and quiesced twenty four hours prior to lysis. The extraction procedure was performed on ice with ice-cold reagents. Cells were washed with PBS and lysed in 500µl buffer A (20mM Hepes, 10mM KCl, 1mM EDTA, 1mM DTT, 100µM sodium vanadate, 10% glycerol, 0.2% NP-40 (v/v)

and Complete™ protease inhibitor tablet). Cell nuclei were sedimented by centrifugation at 10000 rpm for 10 minutes at 4°C and resuspended in 50µl buffer B (20mM Hepes, 350mM NaCl, 10mM KCl, 1mM EDTA, 1mM DTT, 100µM sodium vanadate, 20% glycerol (v/v) and Complete™ protease inhibitor tablet). Nuclei were incubated for 30 minutes on ice and then clarified by centrifugation at 13000 rpm for 10 minutes at 4°C. Nuclear protein concentration was estimated as described in 2.6.

### 2.13.2 Electrophoretic Mobility Shift Assay (EMSA)

The NF-κB consensus oligonucleotide (5'-AGT TGA GGG GAC TTT CCCAGG C-3') and the SP-1 oligonucleotide (5'-ATT CGA TCG GGG CGG GGC GAG-3') were purchased from Promega (Southampton, UK). The NF-κB oligonucleotide (3.5pmol) was labelled with $\gamma$-$^{32}$P ATP (10µCi) with T4 polynucleotide kinase according to the manufacturers instructions (Promega). Binding reactions performed in a total of 10µl contained 5µg nuclear extract, 2µl EMSA binding buffer (50mM Tris-HCl, pH7.5, 20% glycerol (v/v), 5mM $MgCl_2$, 2.5mM EDTA, 2.5mM DTT, 250mM NaCl and 0.25mg/ml poly(dI-dC)•poly(dI-dC) and 1µl $\gamma$-$^{32}$P-labelled probe. Control binding reactions also contained either unlabelled NF-κB, SP-1 oligonucleotides (1.75pmol) or an anti-NF-κB P65 antibody (Santa Cruz, La Jolla, USA). Binding reactions were performed for 20 minutes at room temperature and then resolved on a 4% non-denaturing polyacrylamide gel run in 0.5 x TBE buffer at 200 volts. Gels were transferred to Whatman 3M paper (Whatman Inc, New Jersey, USA), dried on a Biorad Gel-Dryer (Biorad, Hemel Hempstead, UK) at 80°C for 1 hour and exposed to Kodak X-Omat film (Sigma) at -70°C with an intensifying screen. Gels were also exposed to a Molecular Dynamics phospho-screen and analysed on a Molecular Dynamics Storm™ 860 phospho-imager (Amersham Pharmacia Biotech, Amersham, U.K). Band densitometry was performed with the ImageQuant 5.0 software (Amersham Pharmacia Biotech).

## 2.14 ERK Activity Assay

Cells were transfected as described in 2.5 and quiesced twenty four hours prior to lysis. Samples were lysed on ice as for SDS-PAGE in 0.5ml lysis buffer (25mM Hepes (Na salt), 0.3M NaCl, 1.5mM MgCl2, 0.2mM EDTA, 0.5% Triton X-100 (v/v), 20mM β-glycerophoshate, 0.5mM DTT, 1mM sodium vanadate (pH 7.4) and Complete™ protease inhibitor tablet (Boehringer Manheim)). Lysates were cleared by centrifugation at 13000 rpm for 10 min and protein concentration determined as described previously. Protein concentrations were normalised and 400μl of each cell lysate were incubated with 2μg of anti-myc (9E10) antibody (Santa Cruz) for 1 hour at 4°C. Anti-mouse agarose beads (20μl/sample, Sigma) were added and the samples vortexed for 1 hour at 4°C. Agarose beads were washed three times at 4°C in a buffer containing 20mM Hepes, 50mM NaCl, 2.5mM $MgCl_2$ and 0.1mM EDTA and once in kinase buffer containing 20mM Hepes, 0.5mM sodium fluoride, 7.5mM $MgCl_2$, 0.2mM EGTA, 2mM DTT, 10mM β-glycerophosphate, 0.5mM sodium vanadate and Complete™ protease inhibitor tablet. Each sample was incubated at 30°C for 20 minutes in 20μl of kinase buffer containing 10μg myelin basic protein, 100μM ATP and 1μCi $\gamma$-$^{33}$P ATP (500Ci/mmol) (Amersham Pharmacia Biotech). Samples were spotted onto Whatman p81 paper (Whatman Inc, New Jersey, USA) and washed extensively in 0.5% phosphoric acid and allowed to dry, radioactivity was quantified by liquid scintillation counting using 'Flo-Scint' IV (Packard, Biosciences, Groningen, Netherlands) and a Packard 1900 TR liquid scintillation analyser.

## 2.15 Immunoflourescence

Transfected cells were grown on sterile glass coverslips (22 x 22mm, Fisher, Loughborough, UK) placed within 6 well tissue culture plates (Corning, High Wycombe, UK). Cells were washed twice with PBS and fixed with ice cold 70% methanol for 10 minutes followed with a second wash with PBS. Non-specific binding sites were blocked with 0.2% fish skin gelatin/PBS (Sigma) for 20 minutes. Coverslips were probed with anti-myc (9E10) antibody at a dilution of 1 in 50 in

0.2% fish skin gelatin/PBS for 20 minutes. Washed cells were then probed with anti-mouse Alexa-Green 488 (Molecular Probes) conjugated secondary antibody diluted 1 in 400 in 0.2% fish skin gelatin/PBS for 20 minutes. Coverslips were removed from the tissue culture plates, washed in distilled $H_2O$, allowed to dry and mounted on slides using DAKO fluorescent mounting media (DAKO, Ely, UK). Slides were visualised on a Carl-Zeiss Axiovert 5100 microscope (Jena, Germany) and images captured using a Coolsnap cooled CCD camera and OpenLab 3.01 software (Improvision, Lexington, USA).

# RESULTS: CHAPTER 3

# Integrin Affinity Modulation by Ras and Raf

## 3.1 Introduction

Integrin "activation" can be broadly defined as an increase in ligand binding to the integrin receptor. Integrins can use both affinity and avidity modulation to regulate ligand binding. This thesis will concentrate on the mechanisms of integrin affinity modulation and "integrin activation" will refer to integrin affinity unless otherwise stated. Affinity modulation is associated with a conformational change within the integrin that is both a dynamic and regulated cellular process (reviewed in Hynes 1992).

Fibrinogen binding to platelets requires prior activation of the fibrinogen receptor $\alpha_{IIb}\beta_3$, this active integrin is specifically recognised by the PAC1 monoclonal antibody (Shattil *et al.*, 1985). PAC1, a ligand-mimetic antibody recognises ligand binding sites within the active $\alpha_{IIb}\beta_3$ integrin. Integrin-ligand binding is a divalent cation dependent process. Chelation of $Ca^{2+}/Mg^{2+}$ with EDTA at 22°C can inhibit PAC1 binding, while $Mn^{2+}$ has often been used as a general activator of ligand binding to integrins (Abrams *et al.*, 1994; Gailit and Ruoslahti, 1988). Antibodies that recognise Ligand-Induced Binding Sites (e.g. LIBS6 and Ab33) of $\alpha_{IIb}\beta_3$ also activate the integrin by stabilising the active conformation (Frelinger, III *et al.*, 1990).

At present, the mechanisms regulating integrin affinity are poorly understood. A model system in Chinese Hamster Ovary (CHO) cells was developed to allow genetic and pharmacological study of integrin affinity modulation. Cells expressing $\alpha_{IIb}\beta_3$ fail to bind PAC1 even in the presence of activatory stimuli (O'Toole *et al.*, 1990). This suggests that integrin effectors absent in CHO cells may regulate $\alpha_{IIb}\beta_3$ affinity. Substitution of the $\alpha_{IIb}\beta_3$ cytoplasmic domain with $\alpha_5\beta_1$ restored PAC1

binding, other α chain chimeras including $\alpha_2$, $\alpha_{6A}$ and $\alpha_{6B}$ also allowed PAC1 binding (O'Toole *et al.*, 1994). A CHO cell line stably expressing the chimeric integrin $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ (αβ-py cells) was used to screen cDNAs for their ability to suppress the PAC1 binding integrin. An active variant of H-Ras (Ras G12V) was capable of inhibiting PAC1 binding (integrin suppression) to the active chimeric integrin in a MAP kinase dependent manner. Integrin suppression was not restricted to the $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ integrin as other $\alpha_{IIb}\beta_3$ chimeras were also suppressed (Hughes *et al.*, 1997).

Ras expression in several cells types have been reported to alter integrin expression levels. Modulation of integrin levels by Ras appears to be both integrin and cell type specific.

To understand the mechanisms of Ras-mediated integrin suppression, the αβ-py model system will be setup in this laboratory. Subsequent to reproducing initial findings of Ras G12V-mediated integrin suppression, the Ras-ERK pathway will be further examined for its role in integrin suppression.

## 3.2 Detecting affinity modulation in the αβ-py system

To further understand the process of integrin affinity modulation, a model system (αβ-py cells) was developed in CHO-K1 cells. These highly transfectable cells, stably express a chimeric integrin on the cell surface that is used as a reporter for integrin affinity modulation (Hughes *et al.*, 1997).

### 3.2.1 PAC1 antibody binds to αβ-py cells

Integrin affinity status of untransfected αβ-py cells was determined as described in Materials and Methods. Both the primary PAC1 mouse monoclonal antibody and its secondary anti-mouse IgM-FITC (fluorescein isothiocyanate) antibody were titrated for maximal binding to the αβ-py cells. Figure 3.1A shows a representative histogram of FL1-FITC fluorescence against cell counts. Compared to an IgM isotype matched control antibody, the PAC1 antibody binds to untransfected αβ-py cells. Mean fluorescence intensity increased from 6.67 arbitrary units (AU) to 49.29 AU in the presence of the PAC1 antibody for this histogram.

### 3.2.2 Modulation of the integrin by external factors

To show that the chimeric integrin could undergo affinity modulation, cells were treated with 5mM EDTA, 100μM $Mn^{2+}$ or 2μM Ab33. Figure 3.1B shows a representative histogram of five experiments of FL1-FITC fluorescence of PAC1 binding to αβ-py cells in the presence of EDTA or $Mn^{2+}$. In the absence of exogenous mediators, the mean fluorescence intensity of the native integrin was 74.8 ± 8.5 AU. Addition of EDTA led to a loss of PAC1 antibody binding to 16.3 ± 1.4 AU, an increase in antibody binding was observed with 100μM $Mn^{2+}$ (89.8 ± 11.9 AU). The mean fluorescence of PAC1 binding in the three states allows a numerical estimate of integrin activation to be calculated as an activation index as described in Materials and Methods (2.8.2), where 0 units represents an inactive integrin (EDTA) and 100 units an active (high affinity) integrin ($Mn^{2+}$). The activation index (AI) for these αβ-py cells was 80.7 ± 2.3 units. This demonstrates that αβ-py cells

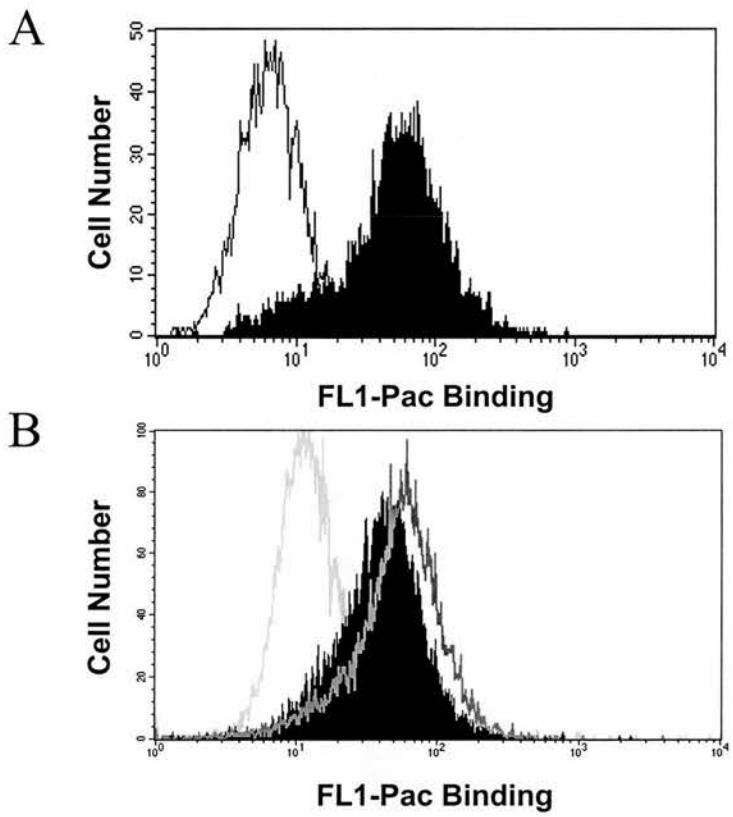


**Figure 3.1 PAC1 antibody binding to αβ-py cells.**

PAC1 antibody binding to αβ-py cells was determined by flow cytometry.

(A) PAC1 antibody binding to αβ-py cells (solid black) and IgM isotype matched control antibody (black line). (B) PAC1 binding in the presence of external factors, untreated (solid black), 5mM EDTA (light grey line) or 100μM $Mn^{2+}$ (dark grey line). Histograms are representative of five experiments.

constitutively express the chimeric integrin $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ in the active state that can be modulated with external mediators.

### 3.2.3 Transfection of αβ-py cells

CHO-K1 cells were chosen as the model system for their high transfection capabilities. Hughes *et al.* (1997) have previously used Life Technologies reagent Lipofectamine, to successfully transfect αβ-py cells. A cell surface marker encoding the extracellular domain of the IL-2 receptor, termed Tac, and the intracellular domain of the $\alpha_5$ integrin (Tac-$\alpha_5$) was used as a marker for transfection. Transfection efficiency of αβ-py cells was detected by flow cytometry using an antibody against the IL-2 receptor, Tac-R-PE (R-phycoerythrin). Figure 3.2A shows that transfection of the Tac-$\alpha_5$ construct into αβ-py cells leads to an increase in Tac-PE fluorescence in the FL2 channel. Compared to an $IgG_1$ isotype matched control antibody, 93% of the cells were positive for Tac-PE antibody binding. The Tac positive cells displayed a range in FL2 fluorescence, representing the degree of transfection with the Tac-$\alpha_5$ construct.

To optimise transfection efficiency, Life Technologies new transfection reagent, Lipofectamine Plus was tested. Figure 3.2B shows that the Plus reagent in combination with Lipofectamine increased transfection from 47.5% to 78.6 %. The Plus reagent was unable to transfect αβ-py cells alone (0.1% transfection). All subsequent transfections were performed with Lipofectamine in combination with the Plus reagent.

The reporter construct Tac-$\alpha_5$ was titrated for maximal expression in αβ-py cells. Transfection of 0.75μg of the Tac-$\alpha_5$ construct was sufficient to obtain a high degree of Tac-PE positive cells (79%) (Figure 3.2C). Higher amounts of the construct were not used so as not to interfere with constructs under test.

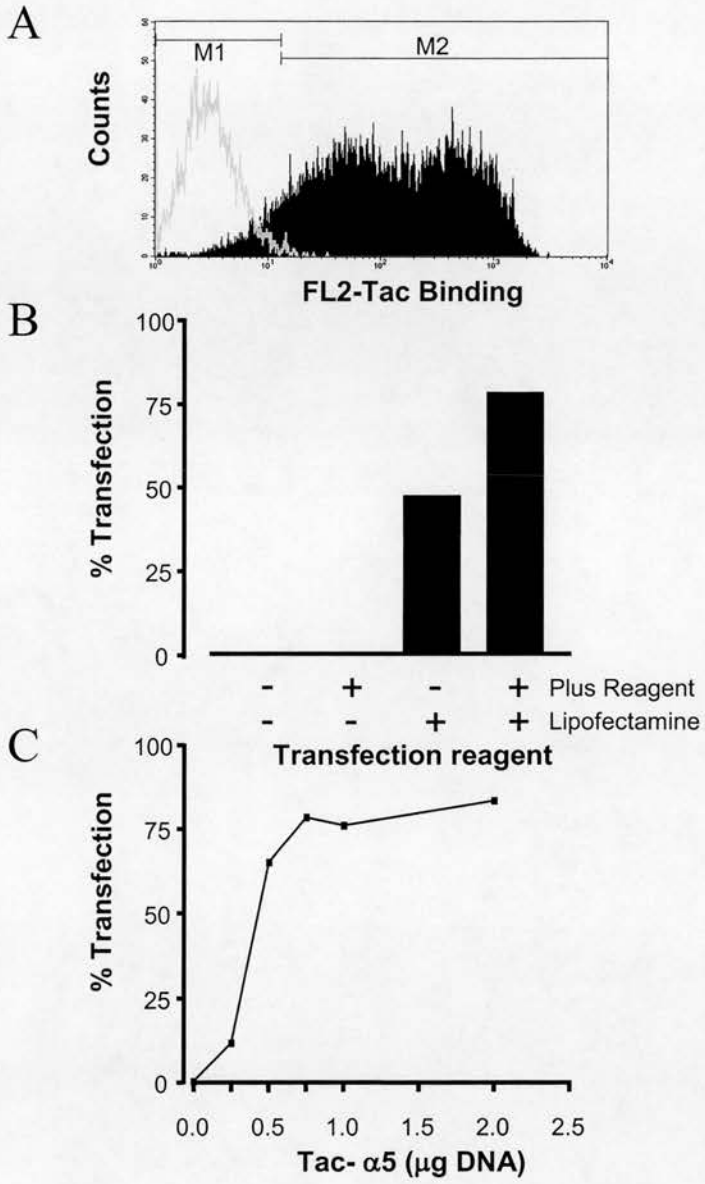


**Figure 3.2 Optimisation of transfection efficiency of the Tac-$\alpha_5$ reporter construct.**

(A) Tac antibody binding to αβ-py cells transfected with 0.75μg Tac-$\alpha_5$ was determined by flow cytometry (solid black) and binding of the $IgG_1$ isotype matched control antibody (grey line). Markers 1 and 2 separate untransfected and transfected cells according to their FL2 fluorescence. Histogram is representative of three experiments. (B) Percentage of Tac positive cells in cells transfected with 0.75 μg Tac-$\alpha_5$ with different transfection reagents and (C) with increasing quantity of the Tac-$\alpha_5$ DNA.

## 3.3 Integrin Affinity Modulation by Ras

### 3.3.1 Ras G12V expression mediates a loss of PAC1 antibody binding

Integrin affinity status in transfected cells was determined as described in Materials and Methods (2.8). The dot blots display PAC1-FITC binding (integrin status) on the x-axis and Tac-PE antibody binding (transfection efficiency) on the y-axis. The quadrant marker on each dot blot differentiates on the x-axis, cells with high and low integrin affinity status and on the y-axis, highly transfected cells (red) against cells transfected to a lesser extent (blue). The quadrant marker separating highly transfected cells was set for individual experiments to contain the top 20-25% of Tac positive cells. Figure 3.3 shows representative dot blots from cells transfected with control vector or the Ras G12V construct. In control vector transfected cells the majority of highly transfected cells (69%) are in the upper right hand quadrant. Addition of 5mM EDTA to the cells leads to a leftward shift in the cell population representing a loss of PAC1 binding with (96%) in the upper left quadrant. Addition of 2μM Ab33 or 50μM $Mn^{2+}$ (not shown) to the cells leads to a shift in the cell population to the right. In the presence of these activating stimuli, the percentage of cells in the high PAC1 binding quadrant increased to (87%). Addition of EDTA or the activating stimuli to the cells act as internal controls for each transfection representing a minimum and maximum of PAC1 binding in these cells. Transfection of the Ras G12V construct into αβ-py cells (Figure 3.3) leads to a decrease in PAC1 binding. This is particularly evident in the highly transfected cells with (68%) in the upper left quadrant compared to only (31%) in control vector transfected cells. Cells with an intermediate degree of transfection show a decreased loss in PAC1 antibody binding. Cells with low transfection efficiency fail to show any decrease in PAC1 binding. PAC1 binding in the presence of either EDTA or Ab33/$Mn^{2+}$ were similar to that observed with the control vector.

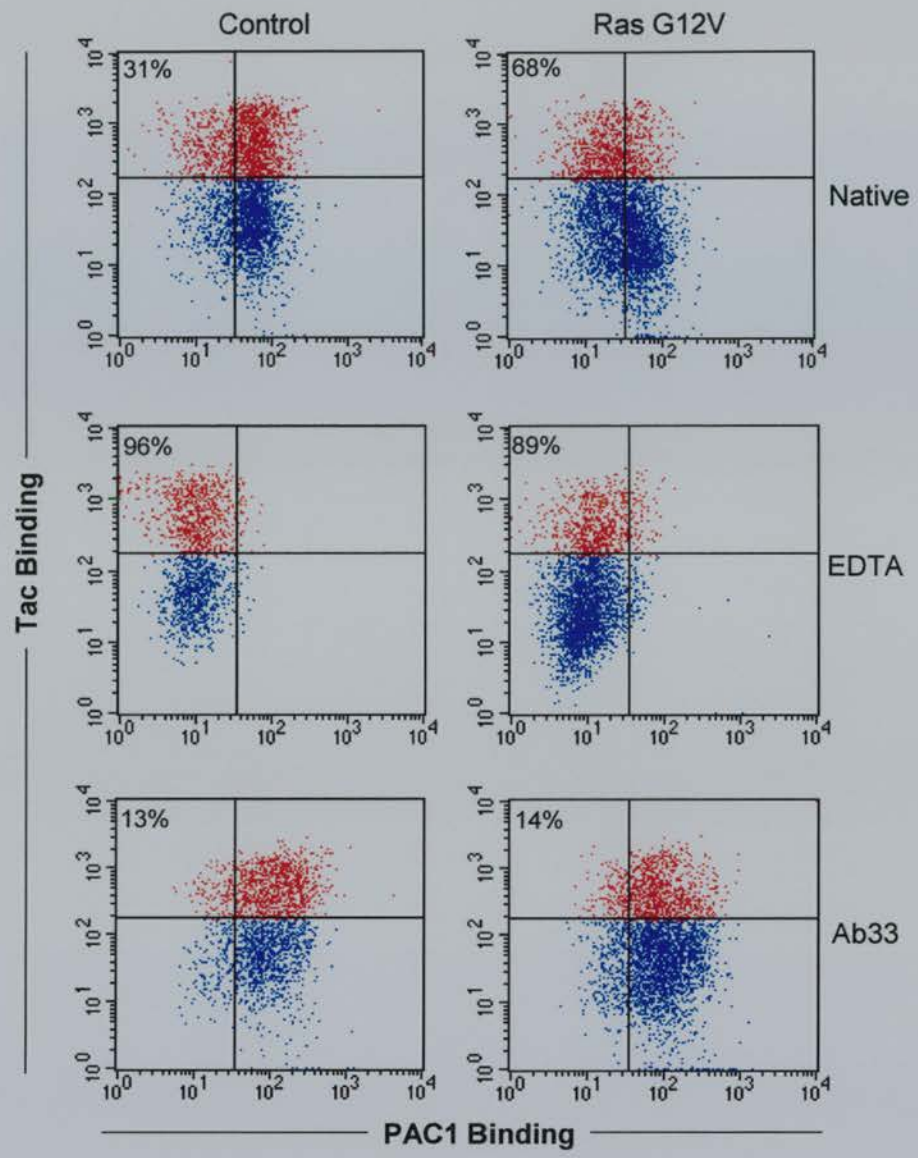


**Figure 3.3 Integrin Affinity Modulation by Ras G12V.**

Flow cytometry was performed on Ras G12V and control vector (1μg) transfected cells to determine integrin affinity. Representative dot blots show PAC1 binding versus Tac binding of Ras transfected cells, high Tac binding cells are shown in red. In addition to the native integrin, PAC1 binding was also performed in the presence of 5mM EDTA or 2μM Ab33. The quadrant markers separate both high and low Tac and PAC1 binding cells. The percentage of high Tac binding cells in the upper left-hand quadrant are shown for each dot blot.

The Ras G12V construct is tagged with the 8 amino acid haemaglutinin epitope (Ha). Expression of the Ha-tagged Ras protein was determined by western blotting of cell lysates prepared from Ras transfected cells with increasing quantity of the Ras G12V construct, total DNA quantity was normalised to 5μg with control vector in each case. Figure 3.4A shows that the Ha-tagged Ras protein runs as a single band at approximately 23 kDa and that 1μg of Ras DNA is sufficient for optimal expression in αβ-py cells. Occasionally a second band is observed in Ras G12V transfected cells with the anti-Ha antibody. This 24-25kDa band has been observed in H-Ras transfected COS cells as a post-translated modified form of the H-Ras protein (Voice *et al.*, 1999).

Examining the activation index in these Ras G12V transfected cells, the reduction in specific PAC1 binding was used to calculate the percentage inhibition relative to the control vector. Figure 3.4B shows that increasing the quantity of Ras G12V DNA led to a rise in the percentage inhibition relative to control cells. This inhibition was maximal (81 ± 3.8%) with transfection of approximately 2μg of Ras G12V DNA.

### 3.3.2 Ras G12V expression leads to ERK1/2 activation

In cells transfected with Ras G12V DNA, activation of the classical Ras-ERK pathway was determined with a phospho-specific antibody that recognises dual phosphorylated ERK 1 and 2. Figure 3.5A shows that in cells quiesced overnight there was an increase in phosphorylation of both ERK1/2 in Ras G12V transfected cells compared to cells transfected with control vector. The degree of phosphorylation was similar in all Ras G12V transfected cells. Total ERK2 levels in all samples were similar and unaffected by Ras expression in these cells (Figure 3.5B).

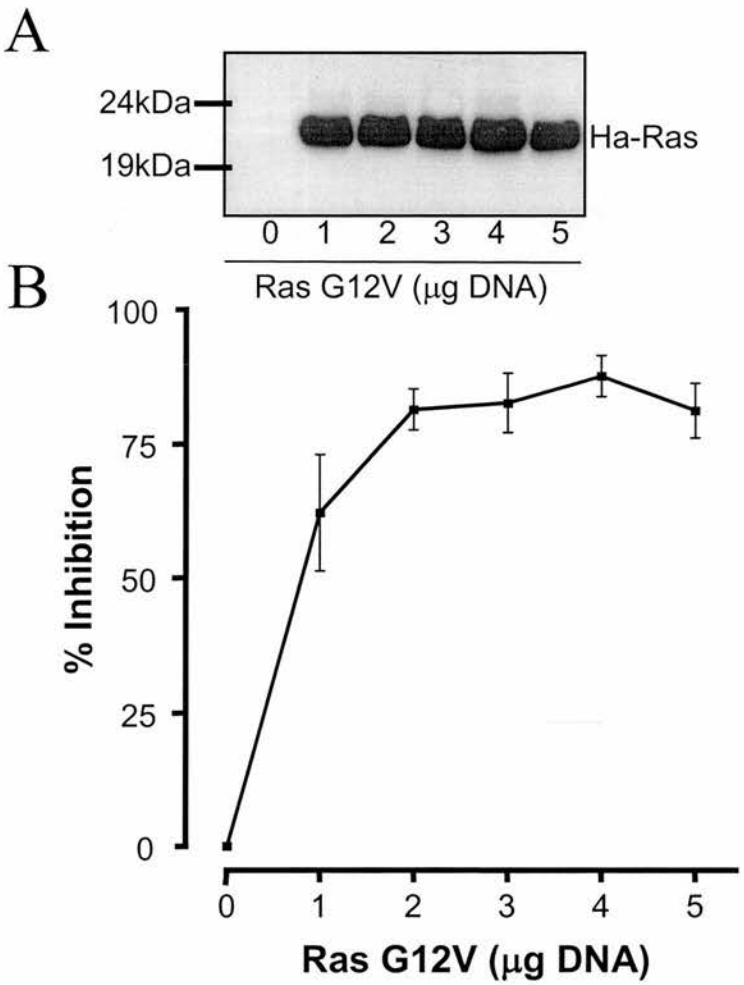


**Figure 3.4 The effect of Ras G12V expression on integrin affinity.**

(A) Lysates from cells transfected with increasing quantity of Ras G12V were probed with the anti-Ha antibody to detect Ras expression. This western blot is representative of three experiments. (B) Suppression of the active integrin was determined in Ras G12V transfected cells. Percentage inhibition was calculated by comparing the activation index in the presence of Ras G12V DNA to that of the control vector. The results shown are the mean ± SEM of 3 independent.

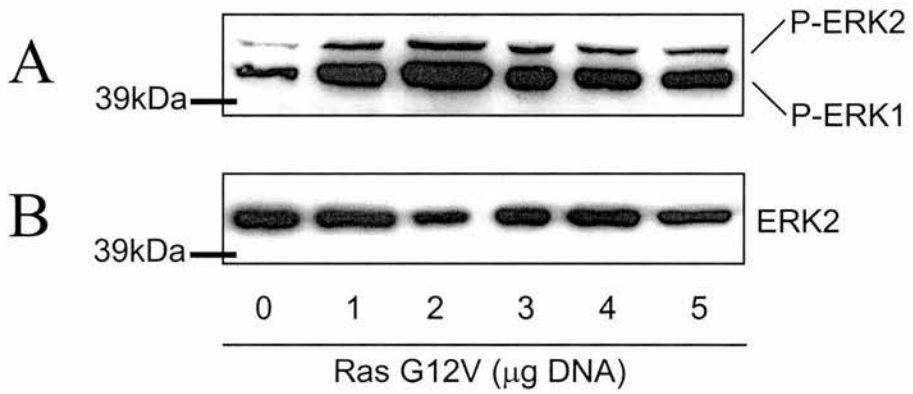


**Figure 3.5 Activation of ERK in Ras G12V transfected cells.**

(A) Ras G12V transfected cell lysates were probed with the phospho-specific ERK antibody and (B) the ERK2 antibody. The western blots are representative of three independent experiments.

## 3.4 Integrin Affinity Modulation by Raf

### 3.4.1 Raf expression mediates a loss of PAC1 antibody binding

The Raf kinase is a downstream effector of Ras. A membrane targeted Raf mutant (Raf-BxB CAAX) that lacks the Ras binding regulatory domain was used as a constitutively active kinase (Daub *et al.*, 1998). Transfection of Raf-BxB CAAX into αβ-py cells displayed a similar loss in PAC1 binding as that oberved with Ras G12V DNA. Figure 3.6 shows that highly transfected Raf-BxB CAAX cells have lost PAC1 binding and that 68% of the cells are now in the upper left hand quadrant compared to 31% for control vector transfected cells. As observed with Ras G12V transfected cells, cells with poor Raf transfection efficiency fail to show any decrease in PAC1 binding.

Expression of the Raf-BxB CAAX construct in αβ-py cells was determined by western analysis of cell lysates. Membranes probed with the anti-Ha antibody identified a protein that runs as a single band at approximately 42-44 kDa (Figure 3.7A). Optimal expression of this construct was seen when 1μg of DNA was used per transfection. Similar expression levels were observed with increasing quantity of DNA.

Figure 3.7B shows the percentage inhibition of the active integrin with respect to increasing Raf-BxB CAAX DNA. Maximum inhibition of the integrin was observed when cells were transfected with 1μg of DNA resulting in an inhibition of 60.6 ± 9.8%. Increasing the quantity of DNA did not significantly increase the level of inhibition (Figure 3.7B).

### 3.4.2 Raf-BxB CAAX expression leads to ERK1/2 activation

The Raf kinase is an an upstream activator of the ERK1/2 pathway. Using the phospho-specific ERK antibody, the phosphorylation state of ERK1/2 was examined in Raf transfected cells. Figure 3.8A shows that in cells quiesced overnight there was an increase in phosphorylation of both ERK 1 and 2 in Raf transfected cells

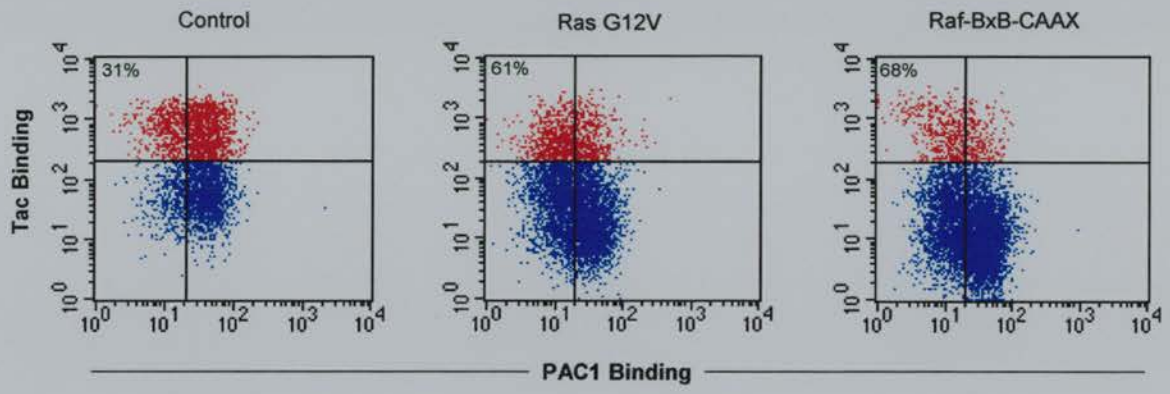


**Figure 3.6 Integrin affinity modulation by Raf.**

Flow cytometry was performed on Raf transfected cells to determine integrin affinity. Representative dot blots show PAC1 binding versus Tac binding of both Ras and Raf transfected cells, high Tac binding cells are shown in red. Cells were transfected with 1μg of DNA in each case. The quadrant marker separate both high and low Tac and PAC1 binding. The percentage of high Tac binding cells in the upper left hand quadrant are shown for each dot blot. The dot blots are representative of three experiments.

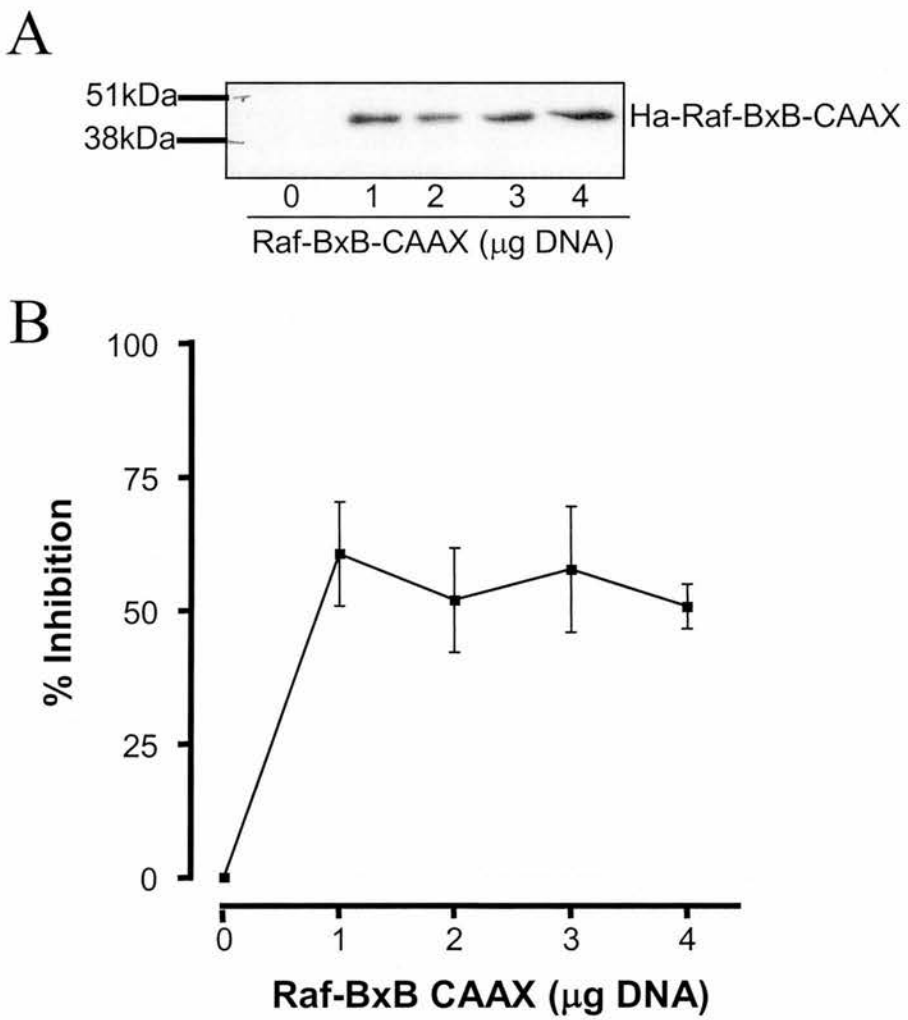


**Figure 3.7 The effect of Raf-BxB CAAX expression on integrin affinity.**

(A) Lysates from cells transfected with increasing quantity of Raf-BxB CAAX DNA were probed with the anti-Ha antibody to detect Raf expression The western blot is representative of three experiments. (B) Integrin affinity of Raf transfected cells was determined by flow cytometry. The results shown are the mean ± SEM of 3 independent experiments.

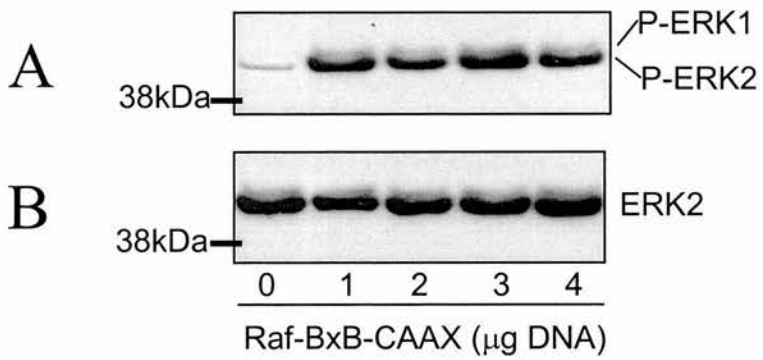


**Figure 3.8 Activation of ERK in Raf-BxB CAAX transfected cells.**

(A) Raf transfected cell lysates were probed with the phospho-specific ERK antibody and (B) the ERK2 antibody. The western blots are representative of three experiments.

compared to cells transfected with the control vector. A comparable increase in phosphorylation compared to control cells was observed in all Raf transfected cell with increasing quantity of Raf DNA. Total ERK2 levels in all samples were similar and unaffected by Raf expression in these cells (Figure 3.8B).

### 3.4.3 Differential effects of Raf isoforms on integrin affinity and ERK1/2 activation

To date three Raf isoforms have been identified, Raf-1, A-Raf and B-Raf. These isoforms have been shown to have differential effects on ERK1/2 activation (Marais *et al.*, 1997), transforming ability and their regulation. Previous results have shown that transfection of Raf-1 into αβ-py cells could mediate a loss of PAC1 binding (Figure 3.7B). It was hypothesised that expression of the Raf isoforms may reveal differential effects on integrin affinity. Using constructs encoding wildtype Raf isofoms, αβ-py cells were transfected and integrin affinity was determined. Figure 3.9A shows that all three Raf isoforms were expressed in αβ-py cells, with molecular weights of 68, 93 and 70kDa for the myc-tagged A, B Raf and Raf-1 isoforms respectively. B-Raf was expressed at a higher level than both A-Raf and Raf-1.

The Raf isoforms have been shown to have different capabilities to stimulate ERK1/2 phosphorylation. Figure 3.9B shows that compared to the empty vector, B-Raf and Raf-1 both displayed an increase in ERK1/2 phosphorylation, with wildtype B-Raf displaying the largest increase in phosphorylation. In contrast, expression of A-Raf did not display any significant increase in ERK1/2 phosphorylation. Expression of ERK2 was unaffected by Raf isoform expression (Figure 3.9C).

Figure 3.9D shows that wildtype Raf-1 can mediate a loss in PAC1 binding thereby suppressing the active integrin. The percentage inhibition by wildtype Raf-1 was 31.2 ± 3.2% compared to 60.6 ± 9.8% for the constitutively active Raf kinase. Increasing expression of Raf-1 protein to levels of B-Raf failed to increase integrin suppression further. Transfection of wildtype B-Raf also inhibits the active integrin to a greater degree than Raf-1 (57.2 ± 2.9%). In comparison to both Raf-1 and B-Raf,

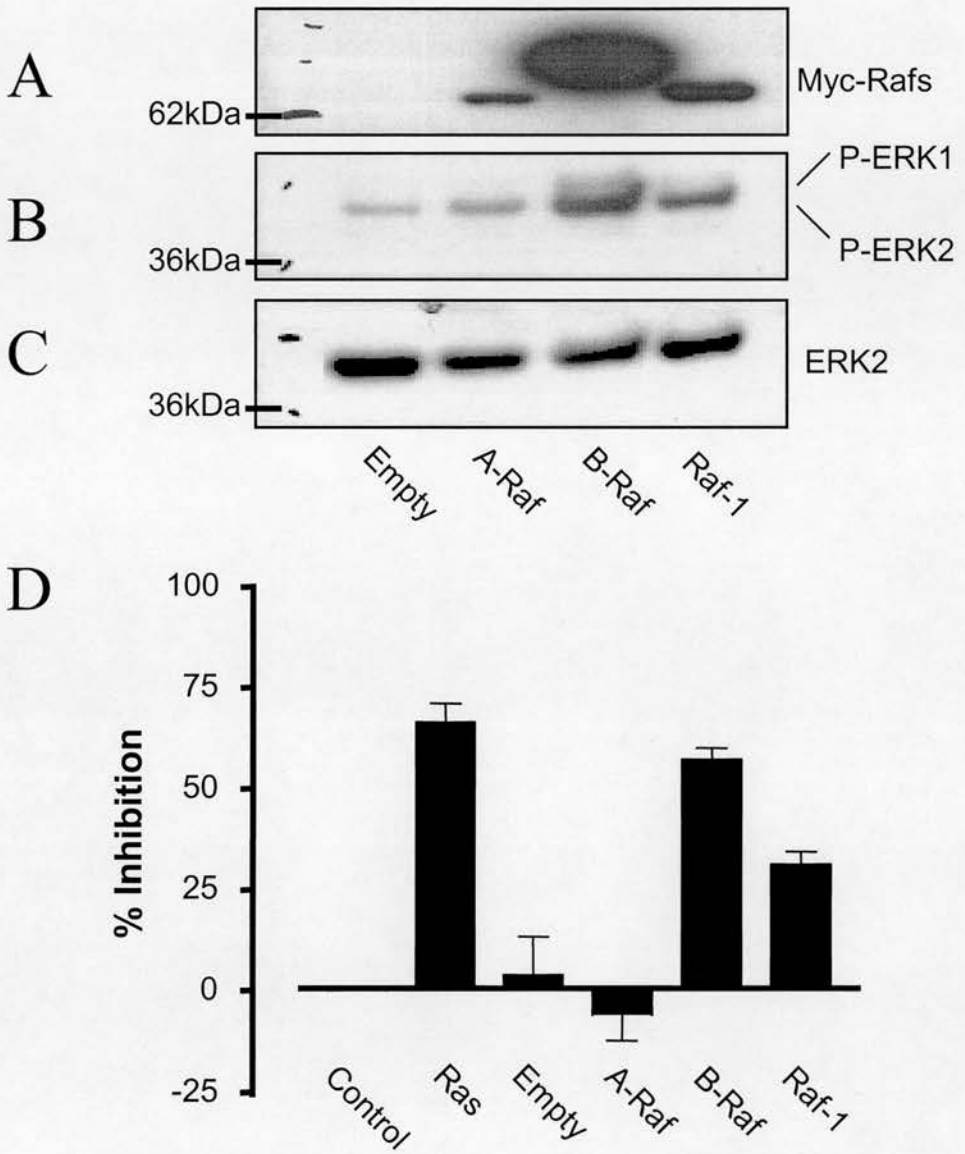


**Figure 3.9 Effect of Raf isoforms on integrin affinity and ERK1/2 phosphorylation.**

Cell lysates from Raf transfected cells were probed with (A) the anti-myc (9E10) antibody. Lysates were also probed with (B) the phospho-specific ERK and (C) ERK2 antibody. These western blots are representative of three experiments. (D) Integrin affinity was determined in Raf transfected cells. The results shown are the mean ± SEM of 3 independent experiments. Cells were transfected with 3μg of Raf isoform DNA/Raf empty vector, 1μg of Ras G12V was used as a positive control.

the Raf isoform A-Raf failed to display any inhibition (-6.1 ± 6.2%). of PAC1 antibody binding

## 3.5 Reversal of Ras-mediated inhibition by MKP-1

To test whether ERK1/2 activation may be required for integrin suppression, integrin suppression was examined in the absence of ERK1/2 phosphorylation. Map kinase phosphatase 1 (MKP-1) is a broad spectrum phosphatase that dephosphorylates both ERK1 and 2 in addition to several other members of the MAPK superfamily (Chu *et al.*, 1996). Transfection of αβ-py cells with a construct expressing MKP-1, which is constitutively active in its wildtype form, reveals a protein of approximately 39kDa by western blotting. A faint band detected with a reduced mobility in MKP-1 transfected cells may reflect a post-translationally modified MKP-1. Co-expression of MKP-1 and Ras G12V in αβ-py cells does not change the level of expression of either MKP-1 or Ras G12V (Figure 3.10). Lysates from these co-expression experiments were also probed with the phospho-specific ERK antibody. Figure 3.10 shows that as previously shown (Figure 3.5), expression of Ras G12V leads to an increase in ERK1/2 phosphorylation. Transfection of MKP-1 into control vector transfected cells leads to a complete loss of ERK1/2 phosphorylation; this was also observed in Ras G12V transfected cells. Following long film exposure times, faint ERK1/2 phosphorylation bands were observed in Ras G12V-MKP-1 transfected cells, however, no bands were observed in control vector transfected cells. ERK2 protein levels in transfected cells remained constant and were unaffected by co-expression of MKP-1 (Figure 3.10E).

To determine whether activation of ERK1/2 was required by Ras G12V for integrin affinity modulation, αβ-py cells were co-transfected with Ras G12V and MKP-1. Figure 3.11 shows that co-transfection of MKP-1 into Ras G12V transfected cells reversed the loss in PAC1 antibody binding observed in Ras G12V transfected cells. Inhibition of the active integrin fell from 51.9 ± 3.7% to 5.5 ± 5.6% in MKP-1 co-transfected cells. Expression of MKP-1 in control vector transfected cells led to a slight increase (activation –11.1 ± 9.7%) in PAC1 antibody binding. This data

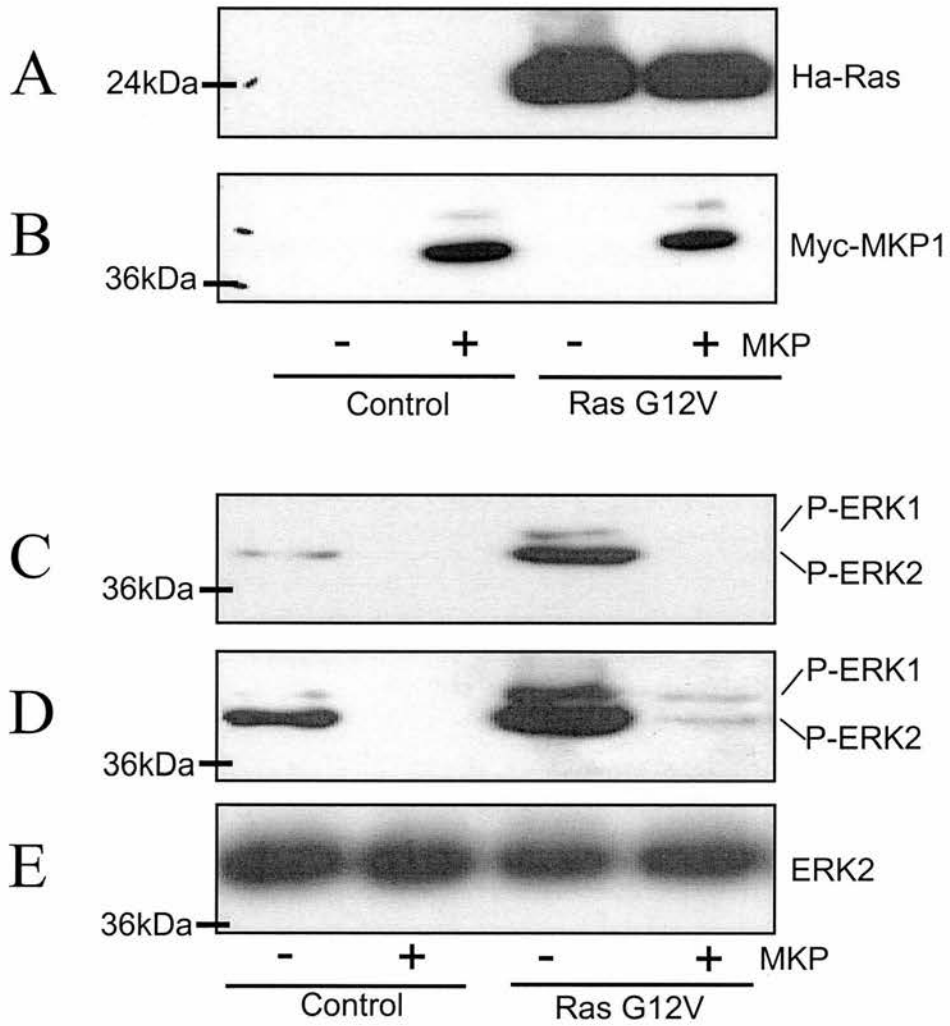


**Figure 3.10 Effect of expression of Ras G12V and MKP1 in co-transfected cells.**

Lysates from cells co-transfected with Ras G12V (1μg) and MKP1 (2μg) were probed with either (A) the anti-Ha antibody or (B) the anti-Myc (A14) antibody. Lysates were also probed for ERK phosphorylation with (C and D) phospho-specific ERK antibody and (D) ERK2 antibody. Westerns blots are representative of three experiments.

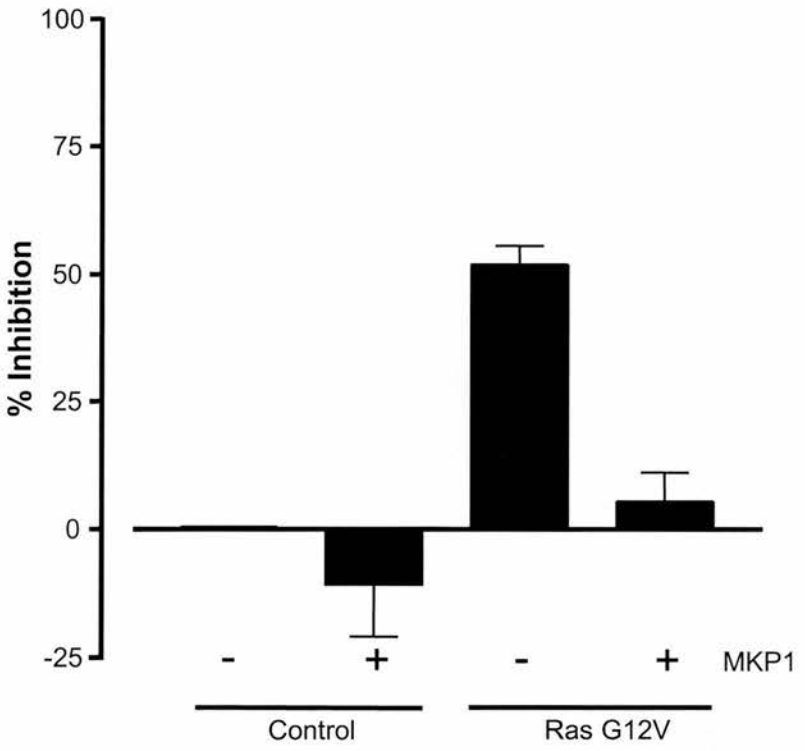


**Figure 3.11 Effect of MKP1 expression on integrin affinity.**

Integrin affinity was determined in Ras G12V (1μg) transfected cells, co-transfected with either MKP1 (2μg) or control vector. The results shown are the mean ± SEM of 3 independent experiments.

indicates that a MAP kinase sensitive to MKP-1 dephosphorylation may be involved in Ras G12V integrin affinity modulation.

## 3.6 Inhibition of MEK1 fails to reverse Ras-mediated integrin inhibition

Downstream of the Raf kinase are the MAPK kinases known as MEK1 and 2. MEK1, the major contributor to ERK1/2 phosphorylation within cells is inhibited by the Parke-Davis compound PD098059. PD098059 is a selective inhibitor of MEK1 with a reported $IC_{50}$ value of 10μM in Swiss 3T3 cells (Dudley *et al.*, 1995).

### 3.6.1 Inhibition of ERK phosphorylation with PD098059

Figure 3.12A shows that PD098059 (10μM) was sufficient to inhibit Ras G12V-mediated ERK1/2 phosphorylation back to control vector levels. At concentrations greater than 10μM, ERK1/2 phosphorylation was reduced further to levels below basal. Pre-treating control cells with PD098059 (100μM) was sufficient to inhibit ERK1/2 phosphorylation, however in Ras G12V transfected cells, a low level of ERK1/2 phosphorylation remained. PD098059 concentrations greater than 100μM were not used as a result of precipitates forming in the culture media.

Treatment of cells with the PD098059 compound did not alter total ERK levels or effect Ras G12V expression levels (Figure 3.12B and C).

Performing band densitometry of the phospho-ERK blot an estimate of the $IC_{50}$ for the PD098059 compound was calculated for inhibition of ERK1/2 phosphorylation in Ras G12V transfected cells. Figure 3.12D shows that the PD098059 compound has an $IC_{50}$ of 12μM, calculated by non-linear regression curve fitting. The $IC_{50}$ value for PD098059 was similar to that quoted in the published literature (Dudley *et al.*, 1995).

Treatment of Ras G12V transfected cells with PD098059 (30μM) for 30 minutes was sufficient to inhibit ERK1/2 phosphorylation. To determine the extent to which

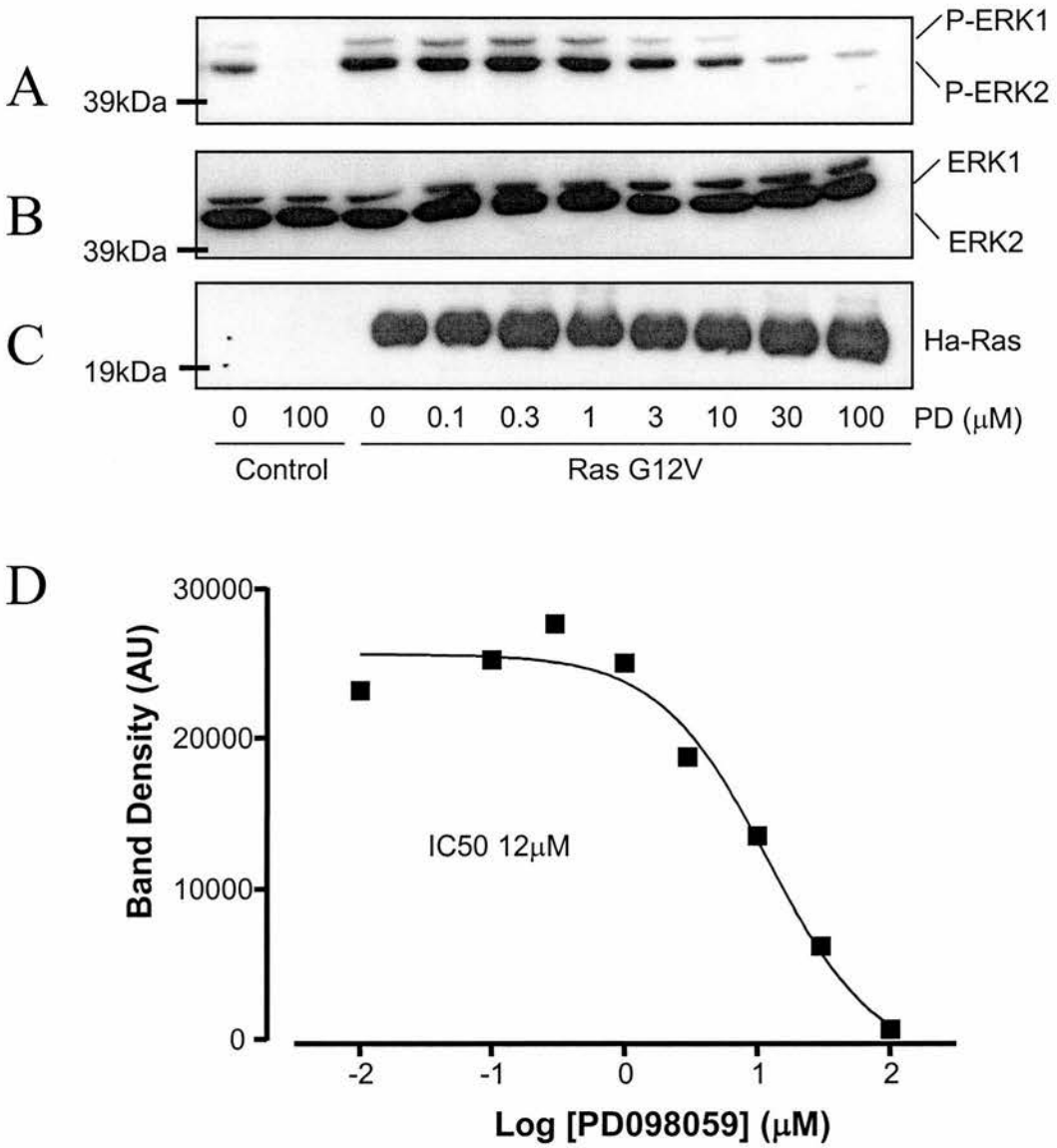


**Figure 3.12 Effect of PD098059 on ERK phosphorylation in Ras G12V transfected cells.**

(A) ERK phosphorylation was determined in Ras G12V (1μg) transfected cells in the presence of increasing concentration of the MEK1 inhibitor PD098059. ERK2 expression levels were also examined in treated cells (B). Expression of Ras G12V was determined with the anti-Ha antibody (C). Western blots are representative of two experiments. (D) Band densitometry was performed on a phospho-ERK blot (Figure 3.12A) using the ERK2 band for analysis. IC50 determination was carried out using non-linear regression curve fitting.

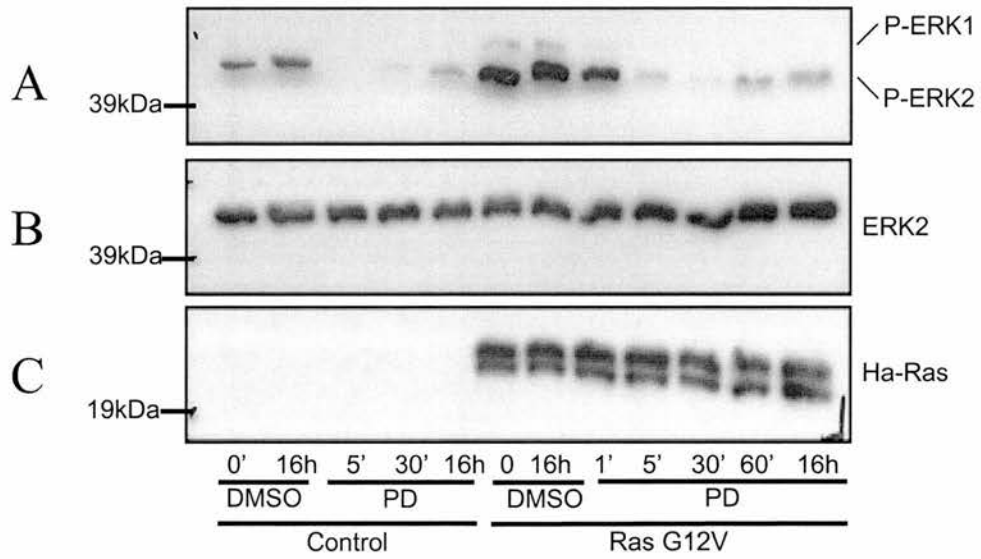


**Figure 3.13 Timecourse of PD098059 inhibition of ERK1/2 phosphorylation in Ras G12V cells.**

Ras G12V (1μg) transfected cells were treated with PD098059 (30μM) (PD) for times ranging from 1min to 16 hours. Lysates were probed with (A) the phospho-specific ERK antibody, (B) ERK2 antibody and (C) anti-Ha antibody.

PD098059 can inhibit ERK1/2 phosphorylation, cells were pre-treated with PD098059 for up to 16 hours prior to cell lysis. Figure 3.13A shows that in Ras G12V transfected cells, inhibition of ERK1/2 phosphorylation occurred within 5 minutes and that phosphorylation levels remained below control levels for up to 16 hours of PD098059 treatment. ERK1/2 phosphorylation was also inhibited in control vector transfected cells for up to 16 hours. ERK2 and Ras G12V expression levels were unaffected by prolonged PD098059 treatment (Figure 3.13B and C). The second band detected by the anti-Ha antibody that runs at approximately 24-25kDa is a post-translationally modified form of H-Ras G12V that has previously been described (Voice *et al.*, 1999).

### 3.6.2 PD098059 fails to reverse Ras-mediated inhibition

Incubating αβ-py cells with 5mM EDTA together with the PAC1 antibody during staining for flow cytometry for 30 minutes is sufficient to inhibit PAC1 binding to the cells. This suggests that affinity modulation of the integrin can occur in a short period of time. Previous results have shown that 30 minute pre-treatment of cells with the PD098059 inhibitor can prevent ERK1/2 phosphorylation in Ras G12V transfected cells (Figure 3.13A). Quiesced cells were treated with PD098059 (30μM) for 30 minutes prior to staining for integrin affinity state determination. Soybean trypsin inhibitor (2mg/ml) was used in this set of experiments, to neutralise the trypsin, rather than serum containing media to avoid complications arising from rapid ERK1/2 activation by serum components. Figure 3.14A shows that Ras G12V transfected cells treated with PD098059 (30μM) displays no change in the percentage inhibition of the active integrin, 65 ± 11.3 and 63 ± 15.5% respectively for diluent and PD098059 treated cells. Statistical analysis (one-way ANOVA) showed no significant difference ($P>0.05$) between either control or Ras G12V transfected cells treated with DMSO and PD098059. Control vector PD098059 treated cells displayed a slight increase in PAC1 antibody binding compared to diluent treated cells (-15 ± 13.4%).

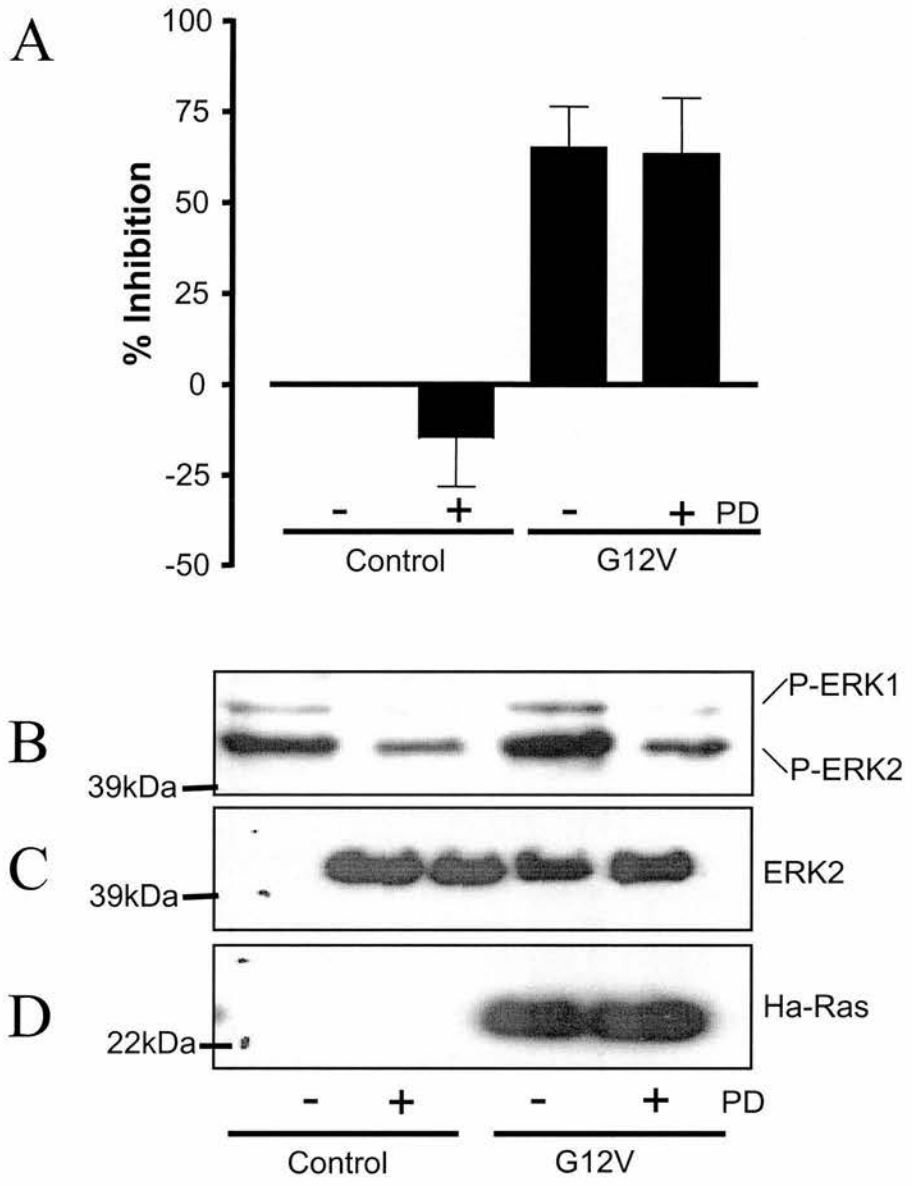


**Figure 3.14 The effect of 30 minute PD098059 treatment on integrin affinity in Ras transfected cells.**

(A) Suppression of integrin activation was determined in Ras G12V (1μg) transfected cells treated with PD098059 (30μM) for 30 minutes. The results shown are the mean ± SEM of 4 independent experiments. Ras G12V transfected cell lysates treated with PD098059 were probed with (B) the phospho-specific ERK antibody, (C) the ERK2 antibody and (D) the anti-Ha antibody. Western blots are representative of three experiments. Statistical analysis was performed by one-way ANOVA test.

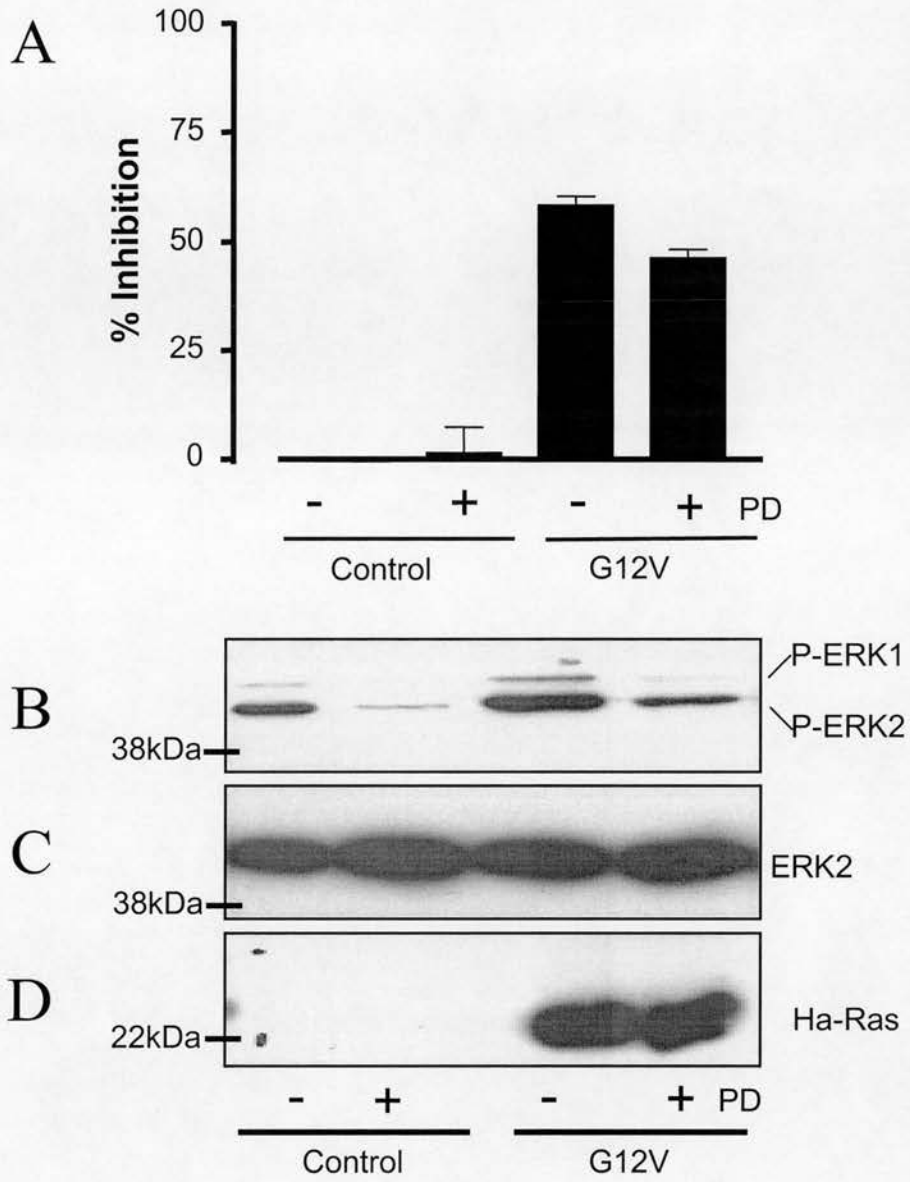


**Figure 3.15 The effect of 16-18 hour PD098059 treatment on integrin affinity in Ras transfected cells.**

(A) Suppression of integrin activation was determined in Ras G12V (1μg) transfected cells treated with PD098059 (30μM) for 16-18 hours. The results shown are the mean ± SEM of 4 independent experiments. Ras G12V transfected cell lysates treated with PD098059 were probed with (B) the phospho-specific ERK antibody, (C) the ERK2 antibody and (D) the anti-Ha antibody. Western blots are representative of three experiments. Statistical analysis was performed by one-way ANOVA test.

Treatment of either Ras G12V or control vector transfected cells with PD098059 (30μM) inhibited ERK1/2 phosphorylation (Figure 3.14B). No change in total ERK2 levels or expression of the Ha-tagged Ras G12V were observed in PD098059 treated cells (Figure 3.14 C and D). The PD098059 inhibitor can prevent ERK1/2 phosphorylation in Ras G12V transfected cells for a sustained period of time (Figure 3.13A). Inhibition of ERK1/2 phosphorylation was observed in cells treated overnight (16-18 hrs). To determine whether reversal of Ras G12V-mediated integrin suppression required a prolonged period of time in the absence of ERK1/2 phosphorylation, cells were treated with PD098059 for between 16-18 hours. Figure 3.15A shows that treatment of Ras G12V transfected cells with PD098059 only produced a slight decrease in integrin suppression, 58.3 ± 2.1 to 46.3 ± 2.0 percent. This drop in percentage inhibition was not statistically significant ($P>0.05$) by the one-way ANOVA test. Treatment of control vector transfected cells with PD098059 showed no change in integrin affinity (1.5 ± 5.9%).

Treatment of either Ras G12V or control vector transfected cells with PD098059 for 16-18 hours inhibited ERK1/2 phosphorylation (Figure 3.15B). No change in total ERK2 levels or expression of the Ha-tagged Ras G12V was observed in PD098059 treated cells (Figure 3.15C and D).

## 3.7 Discussion

The αβ-py transfection and flow cytometry system, developed by Dr. M. Ginsberg and colleagues (Scripps Research Institute, La Jolla, USA), was set up in this laboratory to enable the examination of the role of Ras and its effectors, together with ERK1/2 activation for their role in modulating integrin affinity.

PAC1, a ligand mimetic antibody, specifically recognises the $\alpha_{IIb}\beta_3$ integrin when in the high affinity state (Shattil *et al.*, 1985). The ability of the PAC1 antibody to bind to the chimeric $\alpha_{IIb}\alpha_{6A}\beta_3\beta_1$ integrin present on the αβ-py cells is in agreement with a previous report that this integrin is constitutively active in αβ-py CHO-K1 cells (Hughes *et al.*, 1997). Full length $\alpha_{IIb}\beta_3$ expressed in CHO-K1 cells is not recognised by the PAC1 antibody. It would seem likely that interactions of cell specific factors in CHO-K1 cells to the $\alpha_{6A}\beta_1$ cytoplasmic domain within the chimera must induce a conformation change in the integrin to the high affinity state. Chimeras containing the $\alpha_5\beta_1$ cytoplasmic domain have also been shown to produce a high affinity integrin state (Hughes *et al.*, 1997).

The affinity status of this integrin was modulated through exogenous factors; chelation of $Ca^{2+}$ and $Mg^{2+}$ ions with EDTA at 20-22°C induced a low integrin affinity state, termed "suppressed integrin". Conversely, $Mn^{2+}$ or an $\alpha_{IIb}\beta_3$ stimulating antibody, Ab33, increased the activation index of the chimeric integrin.

CHO-K1 cells are a highly amenable cell line for transfection. Several transfection reagents and electroporation techniques have been used to transfect these cells. Using the Tac-$\alpha_5$ reporter construct, which has previously been shown to have no effect on integrin affinity in αβ-py cells (Chen *et al.*, 1994), we were able to optimise the transfection procedure for these cells in this laboratory. Lipofectamine Plus was used to enhance transfection efficiency to that compared with Lipofectamine, which has previously been used successfully to transfect the αβ-py cells (Hughes *et al.*, 1997). The Plus reagent, a proprietary formulation is a pre-complexing reagent that aids complex formation between DNA and lipid. The Plus reagent in combination with

Lipofectamine increased transfection efficiency by 65% above Lipofectamine alone. In addition to the increased transfection efficiency, the protocol required a reduced quantity of DNA and lipid to achieve the increased level of transfection. Following this protocol, most constructs used throughout this thesis displayed transfection efficiencies between 50 and 80%.

Hughes *et al.* (1997) have previously shown that H-Ras can suppress the chimeric integrin in αβ-py CHO-K1 cells (Hughes *et al.*, 1997). Suppression of the active integrin was not a consequence of a decrease in integrin expression levels as normal PAC1 antibody binding was restored in the presence of an activating $\alpha_{IIb}\beta_3$ monoclonal antibody, anti-LIBS6 (O'Toole *et al.*, 1994). It was also shown that normal PAC1 binding in Ras G12V transfected cells was restored by activating the integrin, indicating that expression of the chimeric integrin was unaffected by Ras expression in αβ-py cells. Fujimoto *et al.* (2001) have recently shown that stable expression of Ras G12V in a pro-B cell line had no effect on the expression of $\alpha_6\beta_1$ (Fujimoto *et al.*, 2001). This correlates with the observations of αβ-py cells transfected with Ras G12V. The chimeric integrin contains the cytoplasmic domain of the $\alpha_6\beta_1$ integrin and therefore signalling events towards this integrin should reflect that observed with the full length $\alpha_6\beta_1$ integrin.

Integrin suppression by Ras was reproduced in this laboratory using a mutant of Ras (G12V) that is constitutively active. Substitution of the glycine at position 12 to a valine disrupts the endogenous Ras GTPase activity; the increase in GTP loading of Ras generates a Ras protein that predominates in the active conformation (Trahey and McCormick, 1987). Ras G12V suppressed the chimeric integrin, represented as a decrease in PAC1 binding. This effect was cell-autonomous as untransfected cells (Tac negative) do not show a decrease in PAC1 binding (Figure 3.3), this suggests that Ras G12V transfected cells cannot affect integrins on neighbouring untransfected cells.

Affinity modulation of integrins by active Ras expression has also been reported by several groups recently. Active Ras has been shown to activate LFA-1 ($\alpha_L\beta_2$) in T-

cells, while in a pro-B cell line, activation of both $\alpha_4\beta_1$ and $\alpha_5\beta_1$ was observed (Liu *et al.*, 1999; Shibayama *et al.*, 1999; Tanaka *et al.*, 1999). These results apparently contradict this data that active Ras mediates integrin suppression. Activation of integrin affinity by Ras has only been observed with integrins in their resting state. Transfection of Ras G12V into CHO-K1 cells expressing full length $\alpha_{IIb}\beta_3$ was shown not to activate the low affinity $\alpha_{IIb}\beta_3$ (Zhang *et al.*, 1996a). The action of Ras on the constitutively active chimeric integrin may reflect differential effects of Ras dependent on the affinity state of the native integrin and the cell type.

Several proteins have been described that bind to Ras, many of these proteins are also activated by the GTP bound form of Ras (Marshall, 1996). One of the best described effectors downstream of Ras is the serine-threonine kinase Raf-1 (Kolch, 2000). Overexpression of the wildtype Raf-1 protein or a constitutively active mutant (Raf-BxB CAAX) both suppressed the chimeric integrin. Activation of Raf-1 has been shown to require binding to membrane localised Ras and activation by the tyrosine kinase, Src (Blumer and Johnson, 1994). Basal Ras and Src activity in cells transfected with wildtype Raf-1 alone, may not sufficiently activate Raf-1 kinase activity in αβ-py cells. Partial activation of Raf-1 in cells transfected with wildtype Raf-1 alone, may reflect the reduction in integrin suppression observed with wildtype Raf-1 compared to Raf-BxB CAAX. The active Raf-1 mutant (Raf-BxB CAAX) suppressed the chimeric integrin to a similar degree to that of Ras G12V. Expression of active Src in αβ-py cells has also been shown to suppress integrins through activation of endogenous Ras and Raf (Hughes *et al.*, 1997). Thus, activation of endogenous Ras and Raf activity can induce suppression of integrin activation.

Expression of either Ras G12V or Raf-BxB CAAX both increased ERK1/2 phosphorylation in αβ-py cells. Dual phosphorylation of ERK2 on amino acids Thr183 and Tyr185 by MEK leads to an increase in ERK kinase activity (Crews *et al.*, 1992). ERK1/2 activation correlated with integrin suppression in both Ras and Raf transfected cells. A similar correlation was observed with the differential activation of ERK1/2 by the Raf isoforms. Suppression was greatest with B-Raf; this kinase displayed increased ERK1/2 activation compared to both Raf-1 and A-Raf.

High basal B-Raf activity has been attributed to the requirement of only Ras interaction for activation (Marais *et al.*, 1997). A-Raf in contrast failed to suppress the integrin and activate ERK1/2. Pritchard *et al.* (1995) have shown that A-Raf is a poor activator of MEK causing reduced ERK1/2 phosphorylation (Pritchard *et al.*, 1995). We cannot exclude the possibility that transfection of wildtype A-Raf into αβ-py cells may not generate an active kinase and explain the lack of integrin suppression. Marais *et al.* (1997) have shown that similar to Raf-1, A-Raf requires both Ras and Src for activation. As wildtype Raf-1 was partially activated in αβ-py cells, demonstrated by the increase in ERK1/2 phosphorylation, the failure of A-Raf to suppress integrins may reflect the lack of ERK1/2 activation, rather than a lack of Raf kinase activity.

The requirement for ERK1/2 activation by Ras and Raf for integrin suppression was addressed by the use of MKP-1 and PD098059. Both MKP-1 and PD098059 prevent accumulation of active ERK1/2 within cells by active Ras either through dephosphorylation of ERK1/2 (Alessi *et al.*, 1993) or inhibition of MEK1 (Dudley *et al.*, 1995). The reduction in ERK1/2 activation by these two methods produced conflicting effects on integrin suppression. MKP-1 reversed Ras G12V-mediated integrin suppression by 89% while prolonged PD098059 treatment only reduced suppression by 20%.

PD098059 is a highly specific inhibitor of ERK1/2 activation (Dudley *et al.*, 1995), at a concentration of 30μM used during these experiments; PD098059 has been shown to have no inhibitory effects on activation of other members of the MAPK superfamily including JNK and p38 MAP kinase (Davies *et al.*, 2000). PD098059 reduces ERK1/2 activation through the stabilisation of inactive MEK1 within the cells (Alessi *et al.*, 1995). The absence of active MEK1 and ERK1/2 in Ras G12V transfected cells treated with PD098059 suggests that neither components downstream of Raf may be required for suppression. PD098059 has also been shown to have no significant effect on adhesion to fibronectin induced by activation of $\alpha_4\beta_1$ and $\alpha_5\beta_1$ by active Ras (Liu *et al.*, 1999; Shibayama *et al.*, 1999). These results also

suggest that a pathway insenisitive to PD098059 may exist for Ras-mediated integrin affinity modulation.

The inability of PD098059 to reverse Ras G12V-mediated integrin suppression may reflect a PD098059 insensitive pathway downstream of Ras. This pathway either diverges at the point of Ras through another Ras effector besides Raf, or that Raf may activate a MKP-1 sensitive kinase pathway that is not dependent on ERK1/2 activation. Both of these proposals will be addressed in subsequent chapters.

The reversal of integrin suppression by MKP-1 may reflect the broad specificity of this enzyme. MKP-1 has shown overlapping substrate specificity with other members of the MAPK superfamily including JNK and p38 MAPK (Chu *et al.*, 1996). Hughes *et al.* (1997) have shown that activation of JNK, by active cdc42, in αβ-py cells does not lead to integrin suppression (Hughes *et al.*, 1997). Inhibition of p38 MAP kinase with SB203580 has also been shown to have no effect on Ras G12V-mediated integrin suppression (P. Hughes personnel communication). This would implicate a MAP kinase other than ERK1/2, JNK or p38 MAP kinase sensitive to MKP-1 dephosphorylation is responsible for Ras G12V-mediated integrin suppression.

In summary the αβ-py integrin affinity detection model and transfection system were set up in this laboratory and that initial findings that active Ras and Raf mediate integrin suppression were reproduced. This suppression while sensitive to MKP-1 co-expression was not affected by treatment with PD098059. This raises the possibility of multiple Ras effectors being involved or that a pathway insentive to PD098059 is required for integrin suppression.

# RESULTS: CHAPTER 4

# Integrin Suppression by Ras Effectors

## 4.1 Introduction

Ras is a small guanine nucleotide binding protein that cycles between an active GTP bound and an inactive GDP bound state. Upon GTP binding, the Ras protein undergoes a conformational change localised to two main areas of primary structure; switch region 1 (amino acid 32-40) and switch region 2 (60-76) (reviewed in Polakis 1993). Mutations of residues within the switch region 1, termed the "effector-domain" have shown a reduction in Ras transforming ability (Sigal *et al.*, 1986; Willumsen *et al.*, 1986). The first Ras effector to be identified was the GTPase activating protein (p120 GAP) (Trahey and McCormick, 1987) subsequently, Raf kinase, PI3-kinase and Ral-GDS were also shown to be effectors (Han *et al.*, 1993; Hofer *et al.*, 1994; Rodriguez-Viciana *et al.*, 1994; Spaargaren and Bischoff, 1994; Warne *et al.*, 1993; Zhang *et al.*, 1993). Other Ras effectors have been identified; see Discussion, (reviewed in Campbell 1998).

Single amino acid mutations within the effector domain have revealed that multiple effector pathways contribute to cell transformation. The best described of which are $Thr^{35}$-$Ser^{35}$ (T35S), $Glu^{37}$-$Gly^{37}$ (E37G) and $Tyr^{40}$-$Cys^{40}$ (Y40C), all within the Ras G12V background. Initial yeast two hybrid and binding studies performed with Ras mutants and effectors Raf, PI3-kinase and Ral-GDS, displayed preferential binding to only one of the effectors while failing to bind to the other two. Ras (G12V, T35S) is able to bind to Raf, Ras (G12V, E37G) to Ral-GDS and Ras (G12V, Y40C) to PI3-kinase (Rodriguez-Viciana *et al.*, 1997; White *et al.*, 1995).

These mutants have routinely been used to ascribe Ras effectors to cell function. Cell transformation has principally been attributed to Raf signalling, however, Ras (G12V, E37G/Y40C) signalling synergised with Ras (G12V, T35S) to enhance

transformation (Khosravi-Far *et al.*, 1996; Rodriguez-Viciana *et al.*, 1997; White *et al.*, 1995). Tumour metastasis, however, appears to rely on Ras-Raf signalling (Webb *et al.*, 1998). These mutants have also been used to analyse Ras function in cell proliferation, DNA synthesis, transcription factor activation and skeletal myogenesis.

In this chapter Ras mutants and their effectors have been used to examine their role in Ras G12V-mediated integrin suppression. Integrin affinity during co-expression studies with Ras mutants and R-Ras G38V will also be performed.

## 4.2 Effect of Ras effector mutants on integrin affinity

In the previous chapter, expression of an active mutant of Ras or Raf has shown to mediate integrin suppression. A MAP kinase pathway was implicated to mediate integrin suppression, however, the MEK1 inhibitor PD098059 failed to reverse Ras G12V-mediated integrin suppression. These results (Chapter3) raise the possibility that Ras effectors in addition to Raf may play a role in integrin suppression. The H-Ras G12V effector mutants in the same expression vector as Ras G12V allow us to examine whether other Ras effector pathways are required for integrin suppression.

### 4.2.1 Activation of ERK by Ras effector mutants

The Ras effector mutations generated in the Ras G12V background were transfected into αβ-py cells. Figure 4.1A shows that all three mutants were expressed in αβ-py cells at approximately 23kDa. Both Ras (G12V, E37G) and Ras (G12V, T35S) mutants displayed similar expression levels to Ras G12V, however a reduced level of expression was consistently observed with Ras (G12V, Y40C).

Probing cell lysates with the phospho-specific ERK antibody showed an increase in ERK1/2 phosphorylation with Ras G12V (Figure 4.1B). Neither Ras (G12V, E37G) nor Ras (G12V, Y40C) transfected cells displayed any increase in ERK1/2 phosphorylation above control vector transfected cells. Compared to control cells, ERK1/2 phosphorylation was slightly increased in Ras (G12V, T35S) transfected cells but not to the same extent as Ras G12V. Total ERK2 levels remained unchanged in Ras transfected cells (Figure 4.1C).

### 4.2.2 Ras effector mutants can mediate integrin suppression

Integrin affinity was determined in cells transfected with the Ras effector mutants. Integrin affinity in Figure 4.2 was represented as an activation index (AI, see Materials and Methods) to highlight the large variability that was observed with Ras (G12V, Y40C). Integrin activation index in control cells (70.7 ± 8.5) fell to 17.4 ± 4.1 in Ras G12V transfected cells (77.0 ± 4.3% inhibition). Both Ras (G12V, E37G)

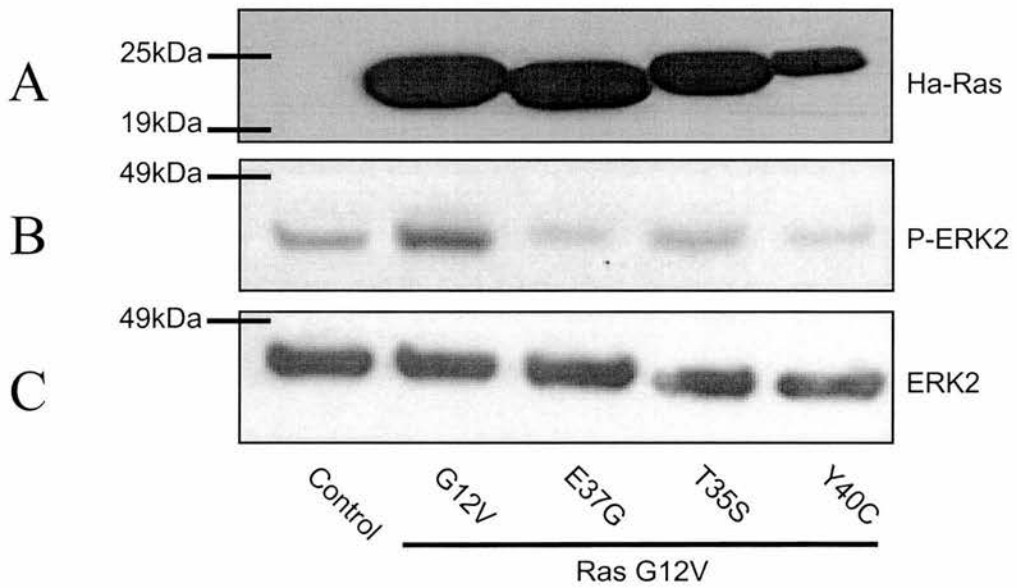


**Figure 4.1 Effect of expression Ras G12V effector mutants on ERK phosphorylation.**

Lysates from cells transfected with the Ras G12V effector mutants (1μg) were probed with (A) the anti-Ha antibody (B) phospho-specific ERK antibody and (C) ERK2. The western blots are representative of three experiments.

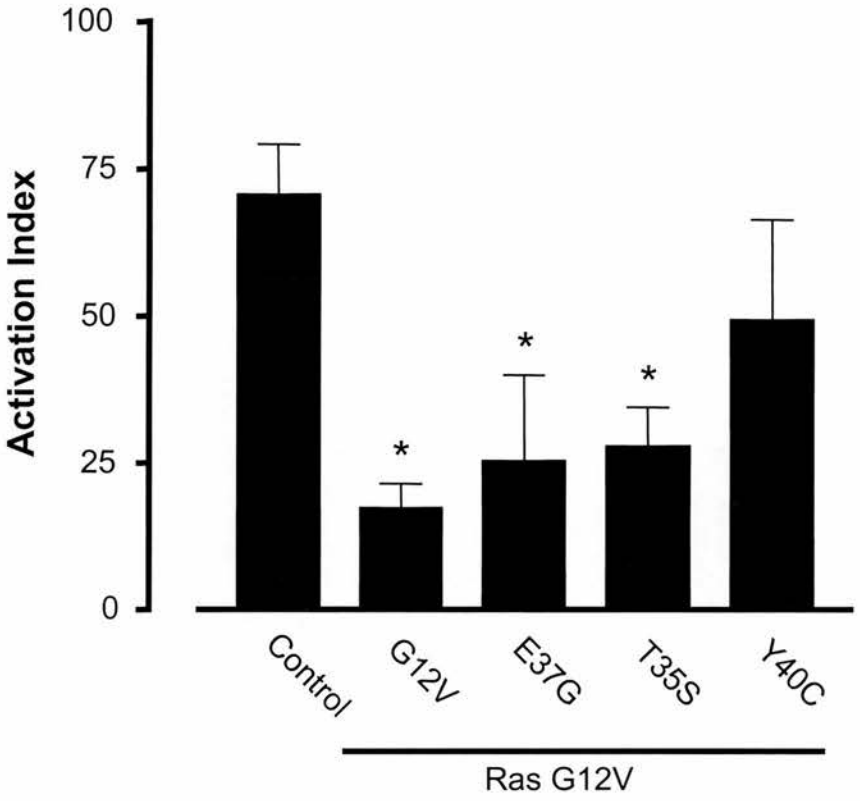


**Figure 4.2 Effect of Ras G12V effector mutants on integrin affinity.**

Activation index was determined in αβ-py cells transfected with Ras G12V (1μg) or Ras G12V effector mutants (1μg). The results shown are the mean ± SEM of 3 independent experiments. Statistical analysis was performed by one-way ANOVA test; asterisk indicates a significant difference ($P<0.05$).

(25.3 ± 14.6) and Ras (G12V, T35S) (27.9 ± 6.6) also displayed a drop in activation index. The Ras (G12V, Y40C) (49.3 ± 17.0) mutant also displayed some integrin suppressive capabilities, however suppression was highly variable between transfections. Except for Ras (G12V, Y40C) transfected cells, all other transfected cells displayed a significant differences in activation index ($P<0.05$) compared to control cells. Increasing Ras (G12V, Y40C) expression to levels similar to Ras (G12V, E37G/T35S) did not increase integrin suppression further (data not shown).

These results suggest that Ras effectors other than Raf may also contribute to integrin suppression, which may explain the inability of PD098059 to reverse Ras G12V-mediated integrin suppression. Individual effector pathways downstream of Ras were subjected to further analysis for their role in integrin suppression.

## 4.3 Integrin suppression by Ras (G12V, T35S)

The Ras (G12V, T35S) mutant has been shown to bind to Raf (White *et al.*, 1995) and increase Raf activity. The increase in Raf activity was approximately 3-4 fold less than Ras G12V (Rodriguez-Viciana *et al.*, 1997). PD098059 failed to reverse Ras G12V-mediated integrin suppression, treating Ras (G12V, T35S) transfected cells with PD098059, the effect of specifically inhibiting the Ras/Raf pathway was determined.

### 4.3.1 PD098059 prevents ERK1/2 phosphorylation

Cells transfected with Ras G12V or Ras (G12V, T35S) were treated with PD098059 (30μM) for 16-18 hours and cell lysates analysed for ERK1/2 phosphorylation. Figure 4.3A shows that both Ras G12V and Ras (G12V, T35S) caused an increase in ERK1/2 phosphorylation compared to control transfected cells. While Ras (G12V, T35S) stimulates ERK1/2 phosphorylation above control vector transfected cells, the increase in Ras G12V transfected cells is far greater. These results were similar to that observed previously (Figure 4.1B). PD098059 inhibited ERK1/2 phosphorylation in all transfected cells. Total ERK2 and Ha-Ras expression were not affected by PD098059 treatment (Figure 4.3B and C).

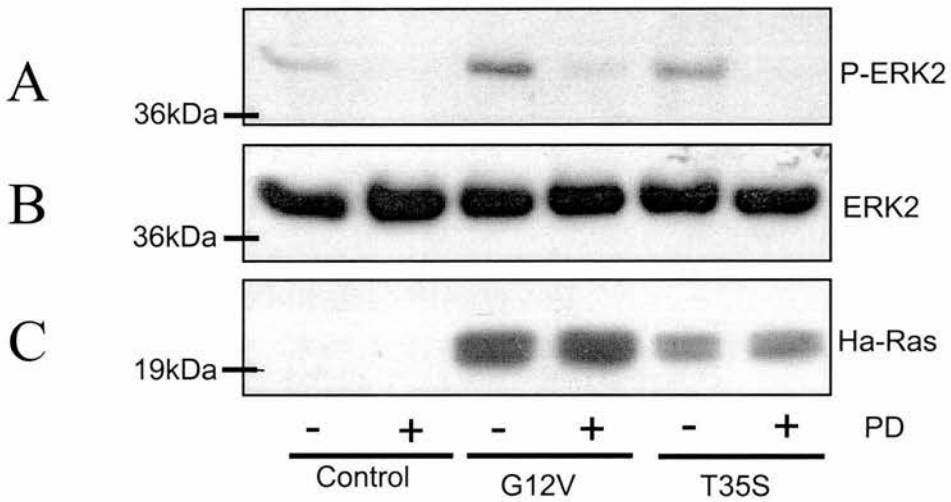


**Figure 4.3 Effect of PD098059 on Ras (G12V, T35S) transfected cells.**

Cells transfected with Ras G12V or Ras (G12V, T35S) (1μg) were treated with PD098059 (30μM) for 16-18 hours. Lysates were probed with (A) phospho-ERK antibody, (B) ERK2 antibody and (C) anti-Ha antibody. Western blots are representative of between 2 and 3 experiments.

### 4.3.2 PD098059 fails to reverse integrin suppression

The increase in ERK1/2 phosphorylation by Ras (G12V, T35S) transfection was inhibited by PD098059 (30μM) treatment. Integrin suppression by Ras (G12V, T35S) however, was only slightly inhibited by PD098059 treatment (56.0 ± 5.5% to 47.3 ± 2.6%). Figure 4.4 shows that the effect of PD098059 on Ras (G12V, T35S) transfected cells was similar to that observed with Ras G12V transfected cells (58.1 ± 1.6% to 42.3 ± 4.3%). Addition of PD098059 to either Ras G12V or Ras (G12V, T35S) was not significantly different ($P>0.05$) to DMSO diluent treated cells. These results suggest that the Ras (G12V, T35S)/Raf pathway may mediate integrin suppression by a PD098059 insensitive pathway.

### 4.3.3 MKP-1 expression prevents ERK1/2 phosphorylation

MKP-1 expression prevented ERK1/2 phosphorylation and reversed integrin suppression by Ras G12V (Chapter 3.5). Whether MKP-1 expression would have a similar effect on Ras (G12V, T35S) transfected cells was determined.

Figure 4.5A and B shows that in cells co-transfected with Ras G12V and MKP-1, both constructs were expressed and that expression levels were similar between co-transfectants. ERK1/2 phosphorylation in Ras G12V and Ras (G12V, T35S) transfected cells was completely inhibited in cells co-transfected with MKP-1 (Figure 4.5C). Total ERK2 levels were unaffected by MKP-1 co-transfection (Figure 4.5D).

### 4.3.4 MKP-1 reverses Ras (G12V, T35S)-mediated integrin suppression

Integrin suppression mediated by Ras (G12V, T35S) was reversed in cells co-transfected with MKP-1 (Figure 4.6). Inhibition fell from 54.6 ± 5.1% to 3.4 ± 9.6% in MKP-1 co-transfectants. The reversal of Ras (G12V, T35S)-mediated integrin suppression was similar to that observed in cells transfected with Ras G12V. MKP-1

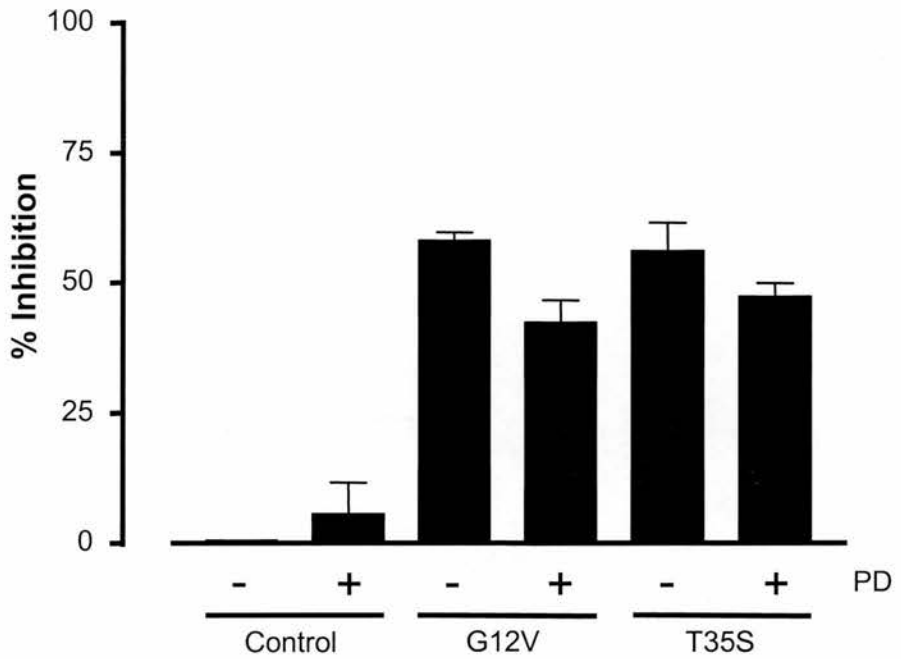


**Figure 4.4 Effect of PD098059 on integrin affinity in Ras (G12V, T35S) transfected cells.**

Integrin affinity was determined in cells transfected with Ras G12V or Ras (G12V, T35S) (1μg) treated with PD098059 (30μM) for 16-18 hours. The results shown are the mean ± SEM of 5 independent experiments. Statistical analysis was performed by one-way ANOVA test.

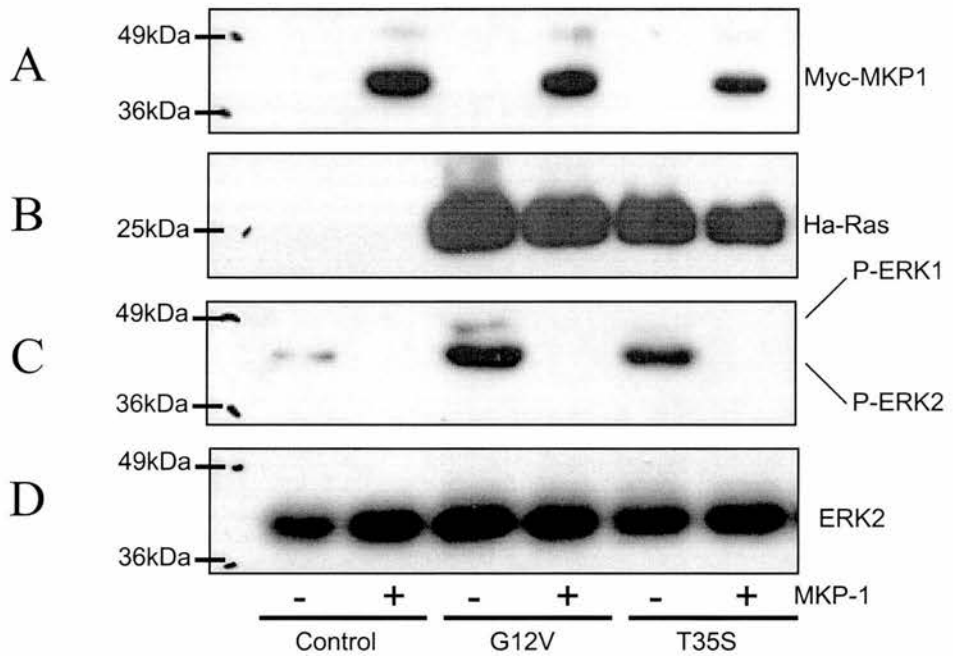


**Figure 4.5 Effect of MKP-1 co-expression with Ras (G12V, T35S) on ERK phosphorylation.**

Lysates from cells co-transfected with Ras (G12V, T35S) (1μg) and MKP-1 (2μg) were probed with (A) anti-myc (A14) antibody and (B) anti-Ha antibody. Lysates were also probed with (C) phospho-specific ERK antibody and (D) ERK2 antibody. The western blots shown are representative of 3 experiments.

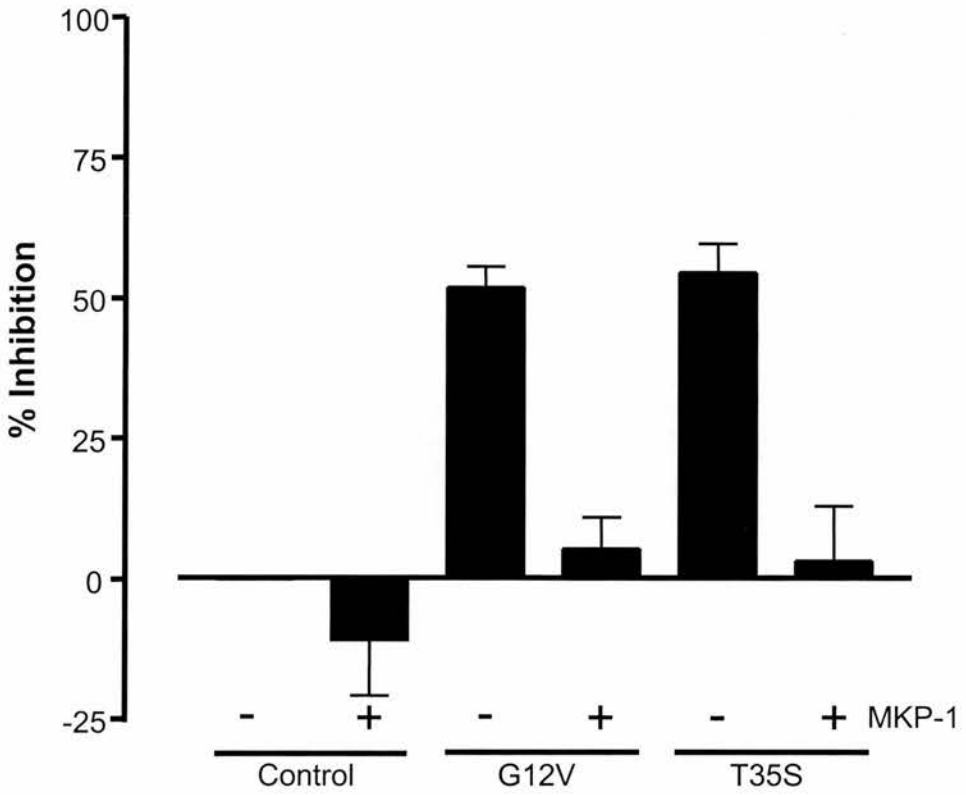


**Figure 4.6 Effect of MKP-1 co-expression on Ras (G12V, T35S) mediated integrin affinity.**

Integrin affinity was determined in cells co-transfected with either Ras G12V or Ras (G12V, T35S) (1μg) and MKP-1/empty vector (2μg). The results shown are the mean ± SEM of 3 independent experiments.

co-expression with the control vector was able to slightly activate the integrin (-11.1 ± 9.7%).

The results observed with cells transfected with Ras (G12V, T35S) mirror those previously observed with Ras G12V. The results suggest that integrin suppression by the Ras (G12V, T35S)/Raf effector arm is mediated by a PD098059 insensitive pathway that remains sensitive to MKP-1 co-expression.

## 4.4 Integrin suppression by Ras (G12V, E37G)

The Ras (G12V, E37G) effector mutant displayed integrin suppressive abilities similar to that of Ras (G12V, T35S). The Ras (G12V, E37G) mutant has been shown to bind specifically to RalGDS and shows no increase in Raf or PI3K activity (Rodriguez-Viciana *et al.*, 1997). This suggests that the pathway downstream of Ras (G12V, E37G) may modulate integrin affinity through RalGDS. RalGDS is a member of three known RalGEFs that increase the activity of Ral downstream of Ras (Albright *et al.*, 1993; Hofer *et al.*, 1994). Using constructs that express constitutively active and dominant negative forms of RalA (bind active RalGEFs and prevent signalling), we assessed the role Ral may play in Ras (G12V, E37G)-mediated integrin suppression.

### 4.4.1 Expression of RalA in αβ-py cells

Initial expression studies with the RalA constructs provided by J. Bos (Netherlands) displayed very poor expression in the αβ-py CHO-K1 cells (data not shown). To increase expression of the Ral constructs, the three Ral constructs (wildtype, 23V (active) and 28N (dominant negative)) were cloned by high fidelity PCR into an alternative mammalian expression vector, pCMV-Tag3B. Cells transfected with the RalA constructs expressed a protein of 26kDa, detected on a western blot with the anti-myc (9E10) antibody (Figure 4.7). All three constructs displayed similar expression levels in the αβ-py cells.

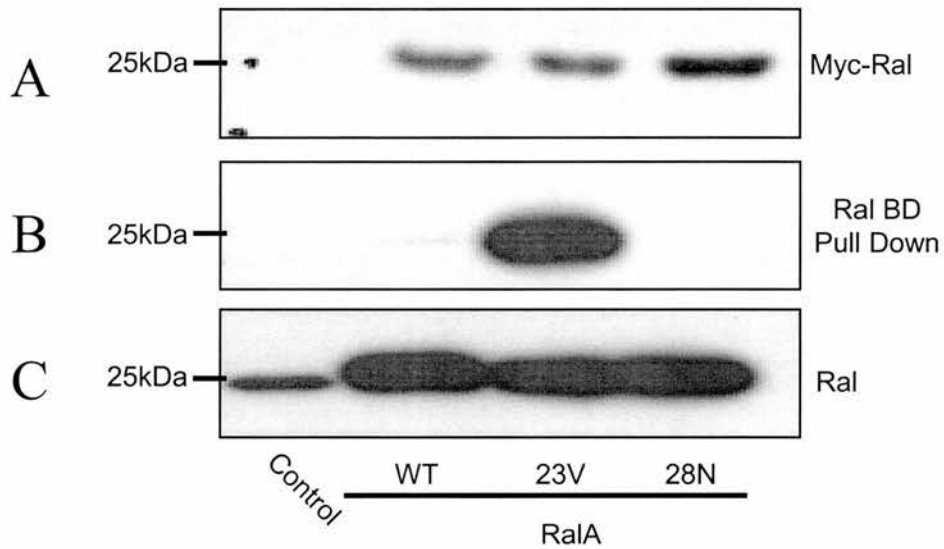


**Figure 4.7 Ral activity of RalA mutants.**

Lysates from cells transfected with either control Tag3B vector or RalA mutants (2μg) were probed with (A) the anti-myc (9E10) antibody. Lysates were subjected to (B) pull down assay with a GST-Ral BD fusion protein. Ral bound to the beads was probed with the anti-Ral antibody. (C) Whole cell lysates were probed with the anti-Ral antibody. Western blots are representative of 3 experiments.

### 4.4.2 Activity of RalA in transfected cells

RalA activity was measured in a pull-down assay with the Ral binding domain of RLIP76 (RalBD), a Ral effector. The amount of Ral precipitated with RalBD has been shown to correlate with the level of GTP bound Ral (Wolthuis *et al.*, 1998). Ral activity was measured in cells transfected with the RalA constructs. Figure 4.7B shows that a small amount of Ral was precipitated in cells transfected with wildtype RalA compared to control 3B transfected cells. Cells transfected with the constitutively active RalA (23V) displayed a large quantity of precipitated Ral, whereas very little was precipitated with the dominant negative RalA (28N). Upon long film exposure times, a small amount of Ral was detected in RalA 28N transfected cells. Whole cell lysates probed with the anti-RalA antibody show that the three RalA constructs were expressed to similar levels. Endogenous RalA was also detected with the anti-RalA antibody in control 3B transfected cells (Figure 4.7C).

### 4.4.3 Effect of RalA expression in Ras (G12V, E37G) transfected cells

The Ras (G12V, E37G) mutant has been shown to increase Ral dependent signalling (Wolthuis *et al.*, 1997). Ral activity was measured in cells co-transfected with Ras (G12V, E37G) and RalA. Whole cell lysates from transfected cells were probed with the anti-RalA antibody (Figure 4.8A). Endogenous RalA expression was not affected with expression of either Ras (G12V, E37G) or RalA constructs. Myc-tagged RalA expression was similar in all transfected cells. Expression of either Ras G12V or Ras (G12V, E37G) led to an increase in endogenous RalA activity compared to control vector transfected cells (Figure 4.8B). An increase in Ral activity was also observed in cells transfected with RalA 23V. The dominant negative RalA construct (28N) inhibited Ral activation in both control and Ras (G12V, E37G) transfected cells. Figure 4.8C shows that expression of Ha-Ras (G12V, E37G) was similar in all transfected cells.

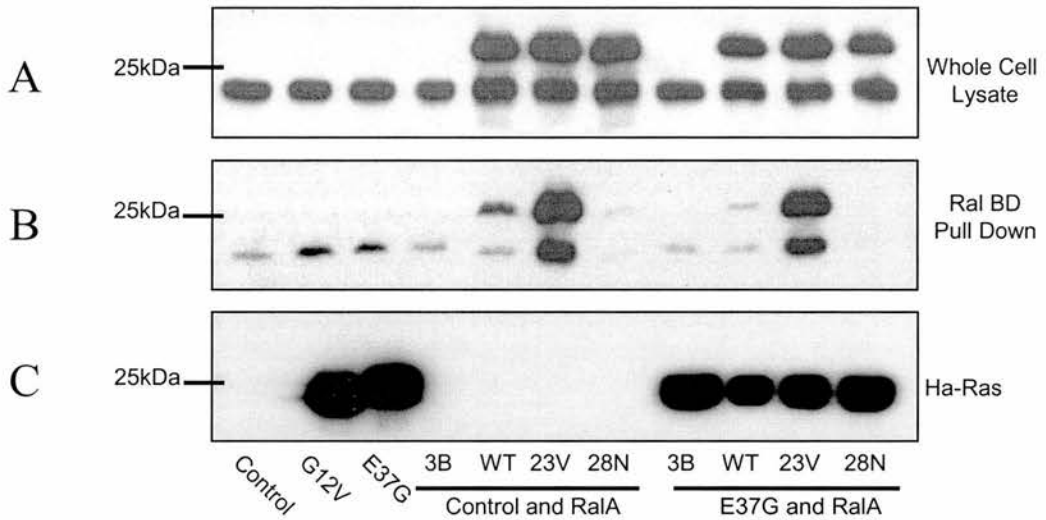


**Figure 4.8 Ral activity in Ras (G12V, E37G) and RalA transfected cells.**

Ral activity was measured in lysates from cells transfected with Ras (G12V, E37G) (1μg) and RalA mutants (2μg). (A) Active Ral bound to GST-Ral BD agarose beads was probed with the anti-RalA antibody. (B) Whole cell lysates were also probed with the anti-RalA antibody and (C) the anti-Ha antibody. The western blots shown are the representative of 3 experiments.

### 4.4.4 Effect of Ras (G12V, E37G) and RalA co-expression on integrin affinity

It was shown that RalA 23V is active in transfected cells and that RalA 28N can prevent Ras (G12V, E37G) activation of endogenous Ral. The effect of constitutively active and dominant negative RalA on integrin affinity is shown in Figure 4.9. Compared to control 3B vector transfected cells, RalA 23V did not lead to any significant suppression of integrins (3.8 ± 7.4%) ($P>0.05$). The dominant negative RalA (28N) only produced a small activation of the integrin (-5.8 ± 10.8%, $P>0.05$). In cells co-transfected with Ras (G12V, E37G), inhibition of Ral activation with RalA 28N only slightly reduced integrin suppression (57.1 ± 0.55% to 47.3 ± 3.3%). Co-expression of Ras (G12V, E37G) and RalA 23V did not increase integrin suppression further than Ras (G12V, E37G) alone (41.6 ± 3.9%). No significant difference in percentage inhibition was observed ($P>0.05$) in cells co-expressing Ras (G12V, E37G) and RalA mutants compared to co-expression with the RalA empty vector.

These results suggest that while Ras (G12V, E37G) can mediate integrin suppression, activation of the Ral pathway may not necessarily be required for integrin suppression.

## 4.5 PI3K activation is not required for integrin suppression

The Ras (G12V, Y40C) displayed inconsistent levels of integrin suppression. This mutant has only been shown to interact with the PI3-kinase p110α and leads to an increase in $PIP_3$ levels (35% of Ras G12V) (Rodriguez-Viciana *et al.*, 1997). Ras (G12V, Y40C) showed no interactions with Raf or RalGDS (Rodriguez-Viciana *et al.*, 1997). To test whether PI3-kinase activation by Ras (G12V, Y40C) was responsible for affects on integrin affinity, a membrane targeted p110α (p110α-CAAX) construct was used.

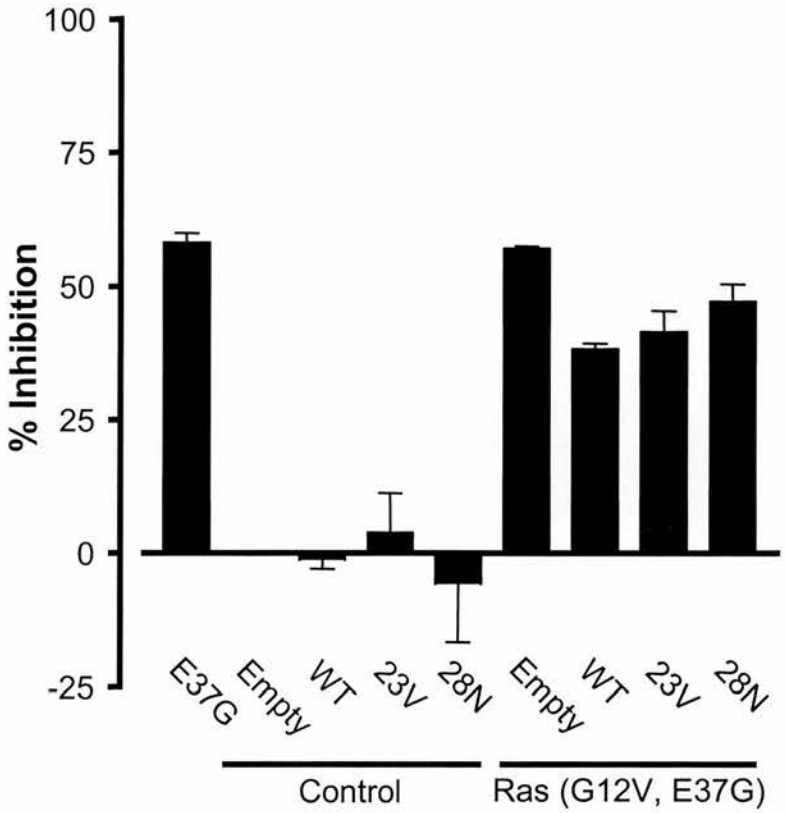


**Figure 4.9 Effect of RalA expression on integrin affinity.**

RalA mutants (2μg) were co-transfected with Ras (G12V, E37G) (1μg) or empty vector. Percentage inhibition was calculated in reference to the Tag3B empty vector. The results shown are the mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA test.

In cells transfected with either Ras G12V or p110α-CAAX, cell lysates probed with an anti-Aktα antibody showed similar expression in all transfected cells (Figure 4.10A). This sheep polyclonal antibody also cross reacts with a protein approximately 36kDa in size, that protein has yet to be identified. An increase in Akt phosphorylation (PI3-kinase effector pathway) was observed in Ras G12V and p110α-CAAX transfected cells in comparison to appropriate control vector transfected cells (Figure 4.10B).

Cells transfected with p110-CAAX were also assessed for integrin affinity. Figure 4.10C shows that cells transfected with p110-CAAX did not lead to integrin suppression (-1.0 ± 10.9%). The cells were responsive to suppressive signals as the positive control Ras G12V was capable of suppressing integrins (57.8 ± 1.0%).

These results suggest that while Ras (G12V, Y40C) may be capable of suppressing integrins, the only known specific Ras effector of this mutant, PI3-kinase is unable to mediate integrin suppression.

## 4.6 Effect of R-Ras G38V expression on integrin affinity

R-Ras, a small GTP binding protein with sequence similarities to the Ras genes has been shown to reverse Ras G12V-mediated integrin suppression (Sethi *et al.*, 1999). Ras G12V-mediated integrin suppression appears to involve several of its downstream effectors. The effect of R-Ras expression on Ras G12V effector mutants (E37G and T35S) was therefore determined. Ras (G12V, Y40C) was not used due to the insignificant integrin suppression observed in Figure 4.1 and with p110-CAAX.

Substitution of a glycine to a valine residue at the corresponding position to the G12V mutation in H-Ras produces a constitutively active R-Ras protein (R-Ras G38V). Lysates from cells co-transfected with Ras G12V effector mutants and R-Ras G38V were analysed for expression of the constructs (Figure 4.11). Expression of the Ha-tagged Ras G12V and the effector mutants was similar to that in Figure 4.1A. The Ras effectors were expressed as a 23kDa protein with similar expression to that of Ras G12V.

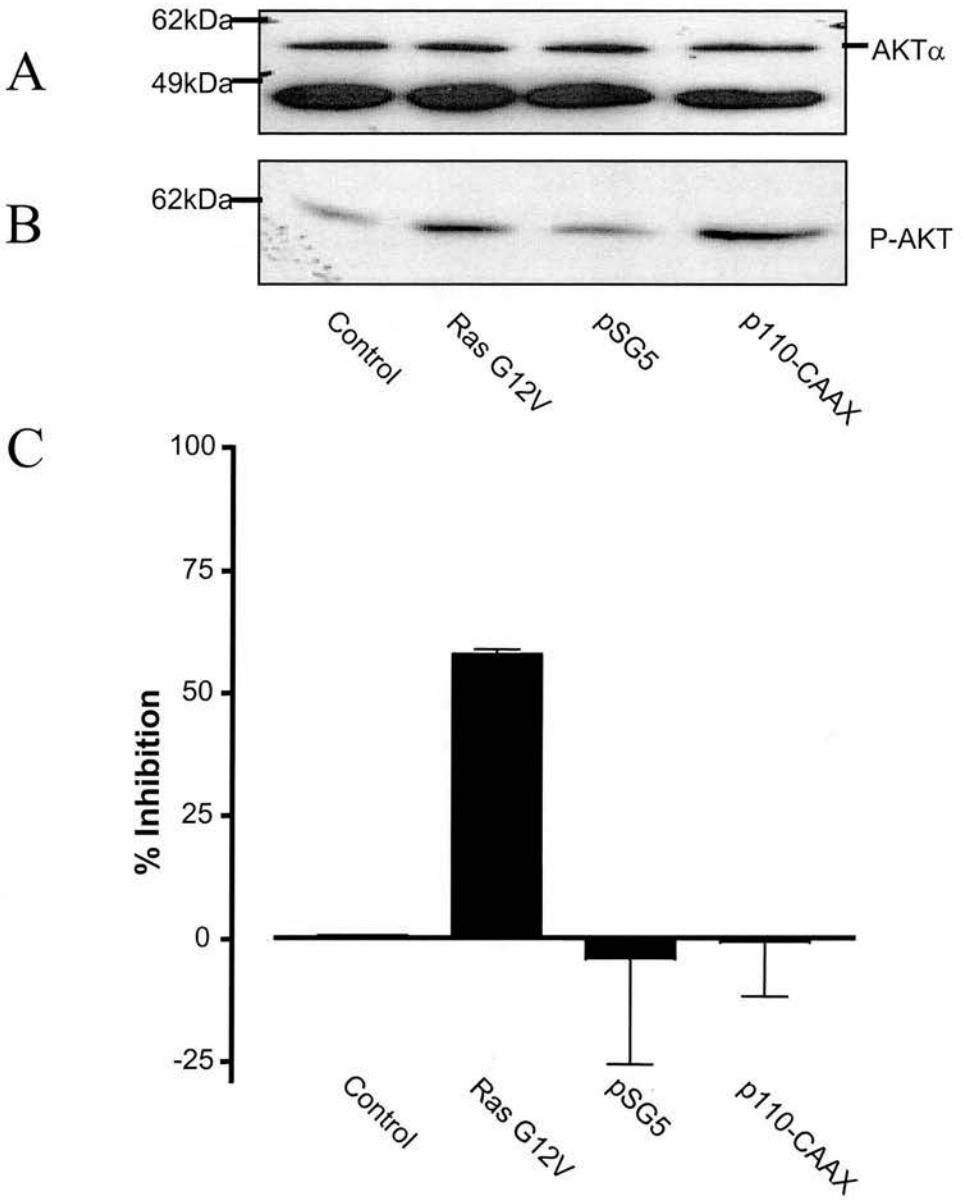


**Figure 4.10 Effect of p110-CAAX expression on Akt phosphorylation and integrin affinity.**

Lysates from cells transfected with Ras G12V (1μg), p110-CAAX (1μg) or appropriate control vectors were probed with (A) phospho-Akt antibody and (B) anti-Aktα antibody. The western blots shown are representative of 3 experiments. (C) Integrin affinity was also determined. Percentage inhibition was calculated in reference to the Ras empty vector. The results shown are the mean ± SEM of three independent experiments.

Figure 4.11B shows that myc-tagged R-Ras G38V was expressed as a 26kDa protein and that expression of this protein was not affected by co-transfection with the Ras G12V effector mutants.

### 4.6.1 R-Ras G38V can reverse integrin suppression by the Ras effector mutants

Transfection of R-Ras G38V into αβ-py cells has previously been shown not to significantly affect integrin affinity of the chimeric integrin (Hughes *et al.*, 1997; Sethi *et al.*, 1999). Transfection of R-Ras G38V displayed a small increase in PAC1 binding (-6.3 ± 2.8%) compared to empty vector transfected cells (Figure 4.11C). As described by Sethi *et al.* (1999), co-expression of R-Ras G38V with Ras G12V reversed Ras G12V-mediated integrin suppression (52.7 ± 6.7% to 5.8 ± 9.9%). The ability of R-Ras G38V to reverse Ras G12V-mediated suppression was mirrored in cells transfected with the Ras G12V effector mutants. Integrin suppression fell from 46.2 ± 4.1% to 3.3 ± 5.2% for Ras (G12V, E37G) and 52.0 ± 1.5% to -3.7 ± 9.1% for Ras (G12V, T35S) in cells transfected in the absence and presence of R-Ras G38V respectively.

These results indicate that the action of R-Ras G38V on integrin affinity may act at a point where the suppressive signals from Ras G12V effector pathways may converge.

## 4.7 Effect of Ras G12V effector mutants on cell morphology

The morphology of a cell is governed by several factors including the arrangement of cytoskeletal components and adhesion strength to substrates. Hughes *et al.* (1997) suggested that suppression of integrins in αβ-py cells transfected with an active Raf might contribute to the round morphology of the cells. We have used the Ras G12V effector mutants to assess whether integrin suppression is responsible for the morphological changes that occur in these cells.

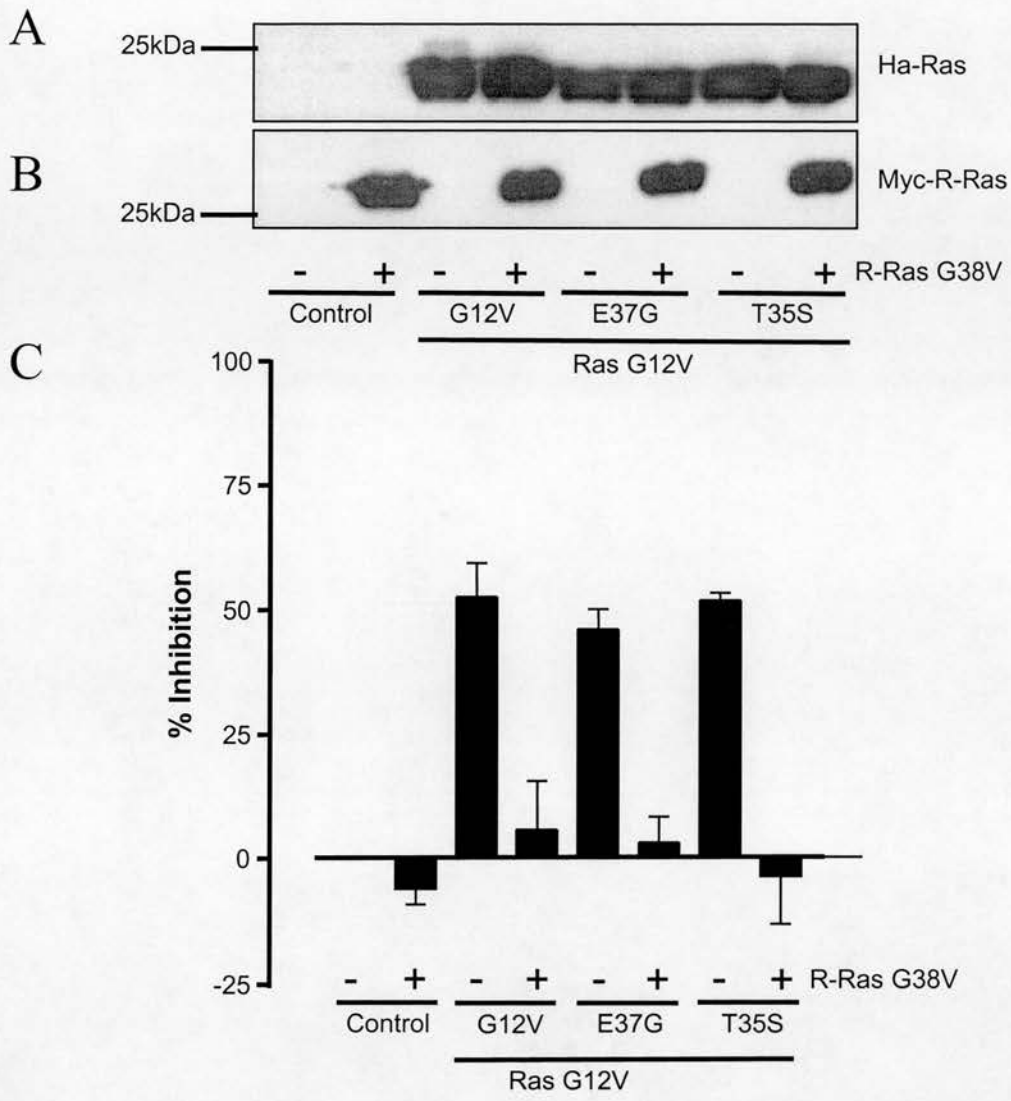


**Figure 4.11 Effect of co-expression of Ras effector mutants and R-Ras G38V on integrin affinity.**

Ras G12V effector mutants (1μg) were co-transfected with R-Ras G38V (1μg) or control vector. Lysates from transfected cells were probed with (A) anti-Ha antibody and (B) anti-myc (9E10) antibody. The western blots shown are the representative of 3 experiments. (C) Integrin affinity was also determined, percentage inhibition was calculated in reference to cells transfected with Ras G12V control vector and R-Ras G38V control vector. The results shown are the mean ± SEM of three independent experiments.

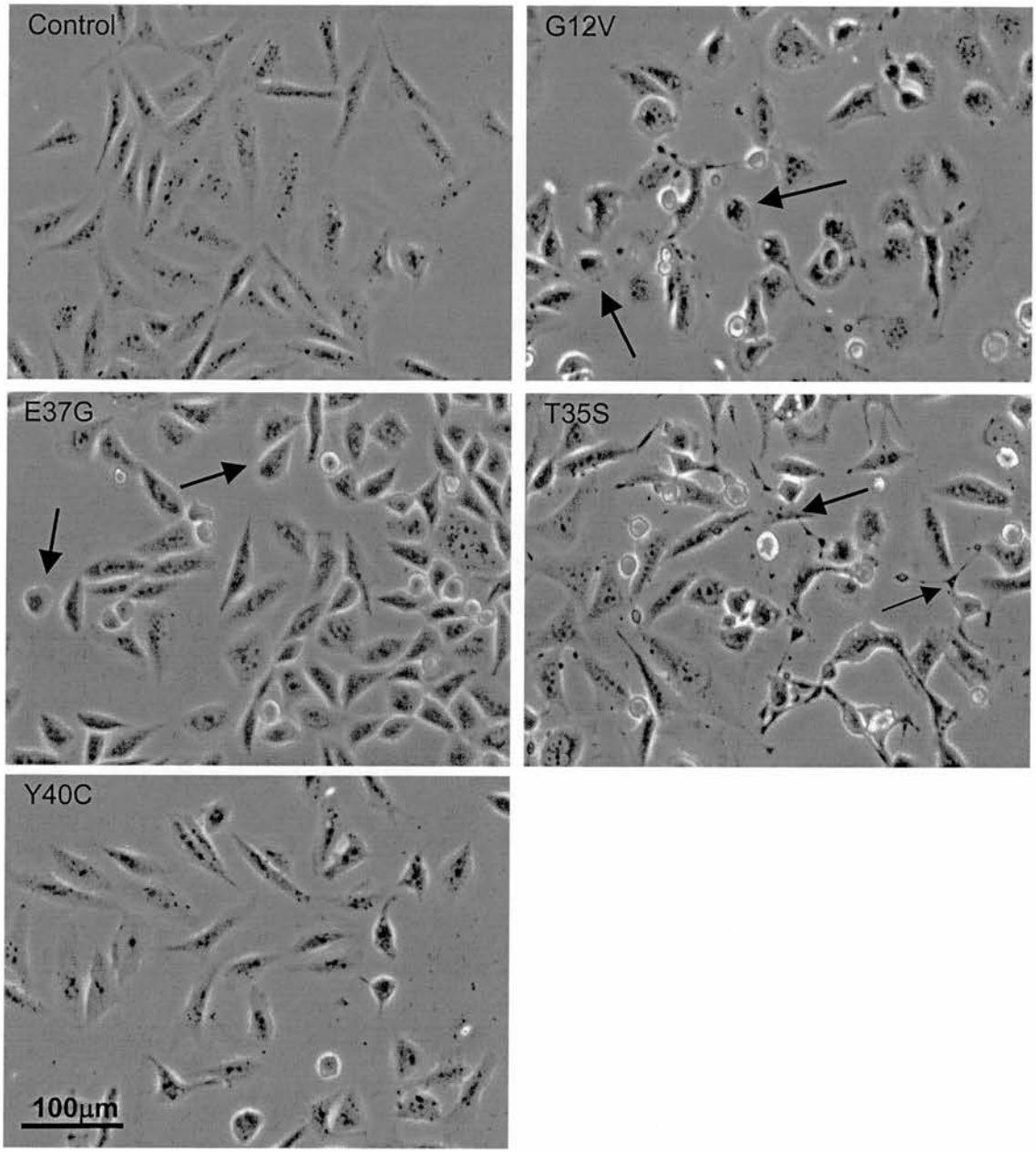


**Figure 4.12 Cell Morphology of Ras effector mutant transfected cells.**

Cells plated on glass coverslips were transfected with Ras G12V or effector mutants (1μg). Forty eight hours post transfection, cell morphology was observed by phase microscopy. Cell morphology pictures are representative of 4 experiments. Scale bar represents 100μm and the arrows represent the described morphology within the text.

Cells grown on glass coverslips were transfected with Ras G12V or the Ras G12V effector mutants. Forty-eight hours after transfection, cell morphology was assessed by phase microscopy (Figure 4.12). Control vector transfected cells displayed a flat, spread phenotype similar to that of untransfected αβ-py cells. In contrast, Ras G12V transfected cells were rounder, more refractile and occasionally possessed long "neurite-like" extensions. Ras (G12V, T35S) transfected cells displayed a similar morphology to Ras G12V transfected cells. Cells were refractile, did not display the spread morphology and had more pronounced "neurite-like" extensions. Cells transfected with Ras (G12V, Y40C) were more akin to that of control vector transfected cells. The cells were flat, non-refractile and did not possess any "neurite-like" extensions. Cells transfected with Ras (G12V, E37G) displayed intermediate cell morphology with slightly rounder cells than control vector cells. These cells however lacked any neurite-like extensions observed with Ras G12V and Ras (G12V, T35S) transfected cells.

Cells transfected with the Ras effector mutants did not significantly differ in cell viability and cell growth during the timecourse of these experiments. The morphological changes observed with transfected cells did not qualitatively increase the number of adherent cells though a detailed assessment of adhesive strength remains to be determined (see Chapter7). Once trypsinised, all cells displayed similar forward and side scatter (size) properties by flow cytometry.

These results suggest that while the Ras G12V effector mutants can suppress integrins, suppression of integrins does not appear to be solely responsible for the round refractile cell morphology.

## 4.8 Discussion

The results presented in this chapter show that individual Ras G12V effector mutants mediate integrin suppression indicating the possibility of multiple pathways being involved. While these pathways utilise different effectors, the suppressive signals may converge at a point where R-Ras can reverse integrin suppression.

Amino acid substitutions within the Ras effector domain have generated Ras mutants that display specificity towards individual Ras effectors (Rodriguez-Viciana *et al.*, 1997; White *et al.*, 1995). The Ras G12V (E37G, T35S and Y40C) mutants have successfully been used to link Ras effector pathways to cell function (Khosravi-Far *et al.*, 1996; Matsuguchi and Kraft, 1998; Rodriguez-Viciana *et al.*, 1997; Webb *et al.*, 1998; White *et al.*, 1995).

Integrin suppression by all three Ras effector mutants suggests that the Ras/Raf pathway is not alone in mediating integrin suppression. Individual Ras effector mutants have shown limited transforming capabilities in certain NIH3T3 lines compared to Ras G12V. Synergism was observed by combining two Ras effector mutants suggesting that complete cell transformation required signals from at least two pathways (Khosravi-Far *et al.*, 1996; White *et al.*, 1995; White *et al.*, 1996). In the case of integrin suppression, Ras (G12V, E37G/T35S)-mediated integrin suppression to almost the same degree as that with Ras G12V. No additional suppression was observed by combining these two mutants. Maximal integrin suppression may therefore only require any one of the three effector pathways.

This hypothesis may explain the inability of PD098059 to reverse Ras G12V-mediated suppression. Inhibition of the Raf/ERK pathway may allow an alternative Ras effector pathway to mediate integrin suppression. However, the failure of PD098059 to inhibit integrin suppression by Ras (G12V, T35S) that only activates Raf argues against a role for MEK and ERK1/2. PD098059 complexes with dephosphorylated, inactive MEK1 and prevents its activation by Raf, possibly allowing Raf to interact with other effectors (Alessi *et al.*, 1995). In systems with high Raf kinase activity (Ras G12V, Raf-BxB CAAX transfected cells), PD098059

may not inactivate all endogenous MEK and therefore low level MEK activity may be sufficient to mediate integrin suppression. In cells treated with PD098059 (Figure 4.3A), ERK1/2 phosphorylation was completely inhibited, however ERK1/2 phosphorylation below our level of detection cannot be ruled out. Results with a constitutively active ERK2 construct are described in Chapter5. The possibility of a MEK/ERK independent pathway downstream of Raf is described further in Chapter5. As with results in Chapter3, MKP-1 expression can still reverse integrin suppression, an ERK-like, protein sensitive to MKP-1 dephosphorylation may therefore act downstream of Raf signalling.

The Ras (G12V, E37G) mutant retained its ability to activate the RalGEFs and in turn to activate Ral. Expression of active RalA (23V) however was insufficient to mediate integrin suppression. Active Ral has been poor in replicating the biological effects of RalGEFs and Ras (G12V, E37G) (Peyssonnaux *et al.*, 2000; White *et al.*, 1996; Wolthuis *et al.*, 1997). It has been proposed that the GTPase deficient RalA (23V) does not mimic the actions of endogenous active Ral as hydrolysis of GTP may be important to activate Ral effectors (Wolthuis *et al.*, 1997). Alternatively RalGEFs may activate Ral-independent pathways that may mediate integrin suppression (Wolthuis *et al.*, 1997). The effects of RalGEFs can be inhibited by dominant negative Ral (Urano *et al.*, 1996; Wolthuis *et al.*, 1997), RalA (28N) had no effect on integrin suppression. Thus, integrin suppression by Ras (G12V, E37G) may not necessarily require RalGEF or Ral activity. RalGEF and Ral independent effects from Ras (G12V, E37G) have also been suggested during skeletal myogenesis (Ramocki *et al.*, 1998).

Phospholipase Cε has recently been identified as a novel Ras effector (Kelley *et al.*, 2001; Lopez *et al.*, 2001; Song *et al.*, 2001). PLCε activity was stimulated by expression of Ras (G12V, E37G) and therefore, effects attributed to Ras (G12V, E37G) will need to take into account of PLCε activation (Kelley *et al.*, 2001). Whether PLCε plays a role in Ras (G12V, E37G)-mediated integrin suppression is currently under study. A PLC inhibitor, U-73122, was shown to inhibit H-Ras-mediated adhesion of Baf3 (haematopoietic cell line) to fibronectin. While H-Ras

G12V has been shown to activate $\beta_1$ integrin affinity in these cells, integrin affinity was not assessed in the presence of the PLC inhibitor (Liu *et al.*, 1999; Shibayama *et al.*, 1998; Shibayama *et al.*, 1999). To address the role of PLCε activation, Ras (G12V, E37G) transfected cells will be treated with U-73122. It should be noted that no data is currently available on the sensitivity of the inhibitor on the PLC epsilon isoform. Alternatively, the Ras (G12V, D38N) effector mutant specifically activates PLCε (Kelley *et al.*, 2001) allowing the role of PLCε to be determined.

PI3-kinase has been implicated in integrin affinity modulation. Activation of LFA-1 ($\alpha_L\beta_2$) and $\alpha_{IIb}\beta_3$ has been observed with active forms of PI3-kinase (Nagel *et al.*, 1998; Zhang *et al.*, 1996b). Expression of Ras (G12V, Y40C) in T-cells caused LFA-1 activation and an increase in binding to ICAM-1 (Tanaka *et al.*, 1999). While expression of Ras (G12V, Y40C) produced inconsistent results towards integrin affinity, active PI3-kinase failed to act upon the chimeric integrin. Inhibition of PI3-kinase activity with LY294002 did not affect Ras G12V-mediated integrin suppression in αβ-py cells (Sethi *et al.*, 1999). The variable nature of Ras (G12V, Y40C)-mediated integrin suppression remains to be clarified; PI3-kinase activity can modulate protein translation through p70 S6-kinase (Dufner and Thomas, 1999) that may explain the poor expression of Ras (G12V, Y40C) compared to the other Ras mutants. The mechanism by which Ras (G12V, Y40C) may mediate integrin suppression remains unclear.

The Ras effectors mutants have implicated several pathways in mediating integrin suppression. Of these effectors, only Raf has been shown to directly modulate integrin affinity. We therefore cannot rule out the possibility that overexpression of the three Ras effector mutants could all lead to Raf activation. While cross talk between the different pathways has not been reported, overexpression of the Ras effector mutants may lead to activation of endogenous Ras (Peyssonnaux *et al.*, 2000). Such a feedback mechanism however, would lead to Raf activation and subsequently ERK1/2 phosphorylation with all three effector mutants. The differential ERK1/2 phosphorylation pattern with the Ras effector mutants suggests

that a global Raf activation may not account for suppression by all the effector pathways.

Growth factors stimulate Ras activation within minutes of treatment (Gibbs *et al.*, 1990). Acute stimulation of Ras activity does not appear to mediate integrin suppression as serum treatment of αβ-py cells has no effect on affinity (data not shown) and that Hughes *et al.* (1997) has shown that integrin suppression occurs approximately 3 hours following Raf activation (Hughes *et al.*, 1997). The events occurring during this 3 hour lag phase remain to be determined.

Ras effectors other than those previously mentioned have been identified (reviewed in Campbell 1998). The RasGAP p120 may act upon the actin cytoskeleton through Rac while MEKK1 can activate JNK (Leblanc *et al.*, 1998; Russell *et al.*, 1995; Yan *et al.*, 1994). Both Rac and JNK have been shown not to affect integrin affinity in αβ-py cells (Hughes *et al.*, 1997). Other effectors include the atypical PKCζ, AF-6 and Rin1 (Diaz-Meco *et al.*, 1994; Han and Colicelli, 1995; Kuriyama *et al.*, 1996). PKCζ has been shown to be required for cytokine stimulated neutrophil adhesion to fibrinogen. This integrin-dependent increase in adhesion was suggested to be a result of avidity modulation of the integrins (Laudanna *et al.*, 1998). AF-6 co-localises with ZO-1 at cell-cell contacts, binding to which is disrupted in the presence of active Ras. The resulting decrease in AF-6 and ZO-1 at cell-cell contact sites may explain the loss of cell-cell contacts in Ras transformed cells (Yamamoto *et al.*, 1997). Rin1, identified by its ability to suppress Ras G12V in yeast (Colicelli *et al.*, 1991) is phosphorylated by the tyrosine kinase c-Abl and the oncogenic fusion protein Bcr/Abl (Han *et al.*, 1997). Co-expression of Rin1 with Bcr/Abl potentiates the oncogenic activity of the Bcr/Abl tyrosine kinase in haematopoietic cells, though this did not require interaction with Ras. Rin1 may therefore act as an effector for Ras and activated tyrosine kinases (Afar *et al.*, 1997). Bcr/Abl expression in haematopoietic cells can activate VLA-5 ($\alpha_5\beta_1$) integrin affinity; the role of Rin1 was not addressed in this paper (Bazzoni *et al.*, 1996). Any one of these effectors could mediate integrin suppression by Ras G12V; it will be interesting to determine their effect on integrin affinity in αβ-py cells.

R-Ras can reverse Ras G12V-mediated integrin suppression at a point downstream of ERK1/2 activation (Oertli *et al.*, 2000; Sethi *et al.*, 1999). The ability of R-Ras G38V to reverse integrin suppression by all three effector mutants suggests that R-Ras may act at a point where these pathways converge. As yet, known effectors of R-Ras have not been shown to modulate integrin affinity, the mechanism of action is therefore unclear. R-Ras is believed to antagonise the suppression pathway rather than a direct activation of the integrin since R-Ras G38V has not been shown to activate the chimeric integrin in αβ-py cells (Sethi *et al.*, 1999).

Ras transformation of cells has often been associated with a change in cell morphology through re-organisation of the actin cytoskeleton by the Rho family of proteins (Khosravi-Far *et al.*, 1995). Integrin suppression did appear to play a role for changes in cell morphology, with cells with suppressed integrins having a rounder morphology than control cells. Cells transfected with Ras (G12V, Y40C) were indistinguishable from control transfected cells. Cell morphology of Ras (G12V, E37G) cells was slightly rounder than control cells this may be a result of the suppressed integrins in these cells. Neurite-like extensions are often observed in Ras transfected neuronal cell lines e.g. PC12 undergoing cell differentiation (Guerrero *et al.*, 1986; Hagag *et al.*, 1986). The presence of such extensions in Ras (G12V, T35S) transfected cells may suggest a similar type of differentiation. Neurite-like extensions by Ras (G12V, T35S) have been observed in transfected non-neuronal cell lines (Khosravi-Far *et al.*, 1996).

In summary integrin suppression by Ras can be reproduced by some of the Ras G12V effector mutants. Integrin suppression can occur by both Raf-dependent and Raf-independent pathways downstream of Ras.

# RESULTS: CHAPTER 5

# Integrin Suppression by Raf-BxB T481A

## 5.1 Introduction

Signalling by Raf is responsible for many of the biological effects attributed to activate Ras. Three isoforms of Raf have been identified and knockout studies in mice have suggested that each have isoform specific *in vivo* functions (reviewed in Hagemann 1999). Common to each isoform is their ability to activate MEK1/2 and subsequently ERK1/2. Activation of ERK1/2 mediates many of the effects of active Raf, including transcriptional changes, cell cycle control and cell transformation (Campbell *et al.*, 1998).

Recent *in vivo* studies have called into question the importance of Raf-1 as an *in vivo* MEK activator. Raf-$1^{-/-}$ mice died during embryogenesis, while Raf-$1^{FF/FF}$ mice (mutation of Raf-$Tyr^{340/341}$, lacks activity towards MEK) survived to adulthood. Cells derived from either strain had normal ERK1/2 activity suggesting that MEK activation by Raf-1 is not essential for development (Huser *et al.*, 2001). The ability of activated Raf to mediate cellular responses in a MEK and ERK1/2 independent manner have been previously described. Differentiation of hippocampal neuronal cells required Raf but not MEK activity (Kuo *et al.*, 1996). Activation of p70 S6 kinase by active Raf was not prevented by co-expression of dominant negative ERK1 or MKP-1 (Lenormand *et al.*, 1996) in CCL39 cells. The interaction of Raf-1 with apoptosis signal-regulating kinase 1 (ASK1) led to an increase in cell survival by a MEK-ERK independent mechanism (Chen *et al.*, 2001). Raf-dependent integrin suppression in the absence of ERK1/2 phosphorylation (Figure 4.4) indicates that Raf may mediate suppression through effectors other than MEK1/2.

Several proteins have been described that have been shown to bind to Raf in addition to Ras and MEK (reviewed in Kolch 2000). Candidate Raf effectors have been

identified including the well described MEK1 protein. Raf phosphorylates cdc25A; the increase in phosphatase activity allowed dephosphorylation of the cyclin dependent kinases and cell cycle progression (Galaktionov *et al.*, 1995). Phosphorylation of the retinoblastoma tumour suppressor protein (Rb) by Raf relieved the inhibitory action of Rb on cell cycle progression at the $G_1$/S boundary (Wang *et al.*, 1998). An interaction between Raf and the anti-apoptotic protein Bcl-2 has been described, leading to localisation of Raf to the mitochondria. Mitochondrial targeted Raf was shown to lead to an increase in BAD phosphorylation (Wang *et al.*, 1996). An ankyrin repeat protein, Tvl-1 was identified as a substrate for Raf phosphorylation, Tvl-1 expression was also shown to potentiate stimulated Raf activity (Lin *et al.*, 1999). Integrin suppression by Raf may reflect activation of a novel effector by Raf.

Mutagenisis of Raf-BxB, a constitutively active variant of Raf (Stanton, Jr. *et al.*, 1989), yielded a Raf mutant unable to bind to MEK (Pearson *et al.*, 2000). Substitution of threonine at position 481 for alanine generated a Raf-BxB mutant that was unable to bind to both MEK1 and MEK2 in a yeast two-hybrid system. Immunoprecipitated Raf-BxB T481A was able to phosphorylate saturating amounts of a MEK substrate *in vitro*, indicating that Raf kinase activity was not significantly affected (Pearson *et al.*, 2000). In this chapter we have used the Raf-BxB T481A mutant to study integrin suppression in the absence of ERK1/2 phosphorylation.

## 5.2 Effect of Raf-BxB T481A mutant on integrin affinity

The downstream Ras effector, Raf-1 has previously been described to suppress the chimeric integrin in αβ-py cells (Figure 3.7). Expression of the Ras (G12V, T35S) Ras effector mutant, displayed integrin suppression via a PD098059 insensitive pathway (Figure 4.4). These results suggest that Raf may mediate integrin suppression through a MEK independent pathway. Utilising a Raf-BxB mutant, T481A, the role of MEK and ERK1/2 activation on integrin suppression was determined.

### 5.2.1 Raf-BxB T481A expression leads to a loss in PAC1 binding

Cells transfected with the constitutively active Raf mutant, Raf-BxB CAAX, displayed a loss in PAC1 binding and suppression of the active integrin. Figure 5.1A shows that the Raf-BxB T481A mutant also leads to a loss of PAC1 binding compared to control vector transfected cells. Representative dot blots show that 72% of the highly transfected Raf-BxB T481A expressing cells were in the upper left hand quadrant of the dot blot compared to 35% for control cells. Dot blots for Raf-BxB CAAX and T481A displayed similar shifts in cell populations to the low PAC1 binding quadrant and as previously described, cells transfected to a lesser extent displayed a reduced degree of integrin suppression.

Cell lysates from Raf transfected cells were probed for expression of the Raf constructs. The Ha-tagged Raf-BxB CAAX protein ran with a mobility of approximately 42-44 kDa (Figure 5.1B). The Ha antibody also detected a second band with increased gel mobility. This band was not a degradation product of the Raf-BxB CAAX protein as it was also present in Raf-BxB T481A and control vector transfected cells. This non-specific Ha antibody interaction was occasionally observed with high protein loading of the gels. The Raf-BxB T481A protein ran with an estimated size of 37kDa and was detected with an anti-Raf antibody (E-10) that recognises a C-terminal epitope (Figure 5.1C). Raf-BxB CAAX was not recognised

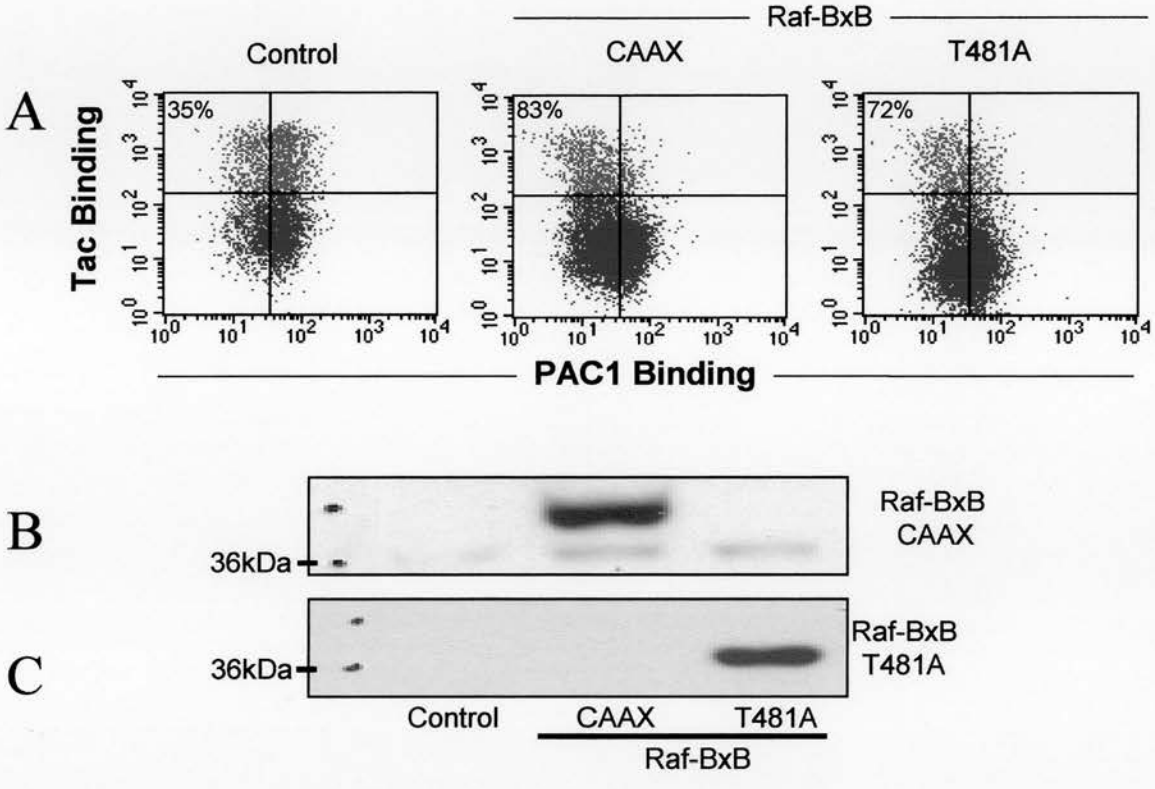


**Figure 5.1 Integrin affinity modulation by Raf-BxB mutants.**

(A) Flow cytometry was performed on Raf-BxB transfected cells to determine integrin affinity. Representative dot blots show PAC1 binding versus Tac binding of Raf-BxB CAAX (1μg) and T481A (1μg) transfected cells. High Tac binding cells are shown in red. The quadrant markers separate both high and low Tac and PAC1 binding. The percentage of high Tac binding cells in the upper left hand quadrant is shown for each dot blot. Dot blots are representative of 3-5 experiments. (B) Cell lysates from transfected cells were probed with the anti-Ha antibody and (C) Raf antibody, E10. The western blots shown are representative of 4 experiments.

by this antibody possibly due to the presence of the CAAX motif that may disrupt the C-terminal epitope recognised by this antibody.

Inhibition of the chimeric integrin was examined by flow cytometry of Raf transfected cells. Figure 5.2A shows that Raf-BxB T481A was able to inhibit PAC1 binding by 60.7 ± 4.6% relative to control vector transfected cells. Cells transfected with either Ras G12V or Raf-BxB CAAX displayed inhibition of 67.8 ± 5.4% and 75.6 ± 6.5% respectively.

### 5.2.2 Raf-BxB T481A fails to activate ERK1 and 2

The T481A mutation of Raf-BxB has been described to prevent binding of MEK1 to Raf *in vitro* (Pearson *et al.*, 2000). Cell lysates from αβ-py cells transfected with Raf-BxB T481A were analysed for the presence of phosphorylated ERK1 and 2. Figure 5.2B shows that expression of Raf-BxB T481A failed to increase ERK1/2 phosphorylation compared to control vector transfected cells. Expression of either Ras G12V or Raf-BxB CAAX in contrast produced a substantial increase in ERK2 phosphorylation. ERK2 expression levels in all transfected cells were similar and independent of the Raf construct used (Figure 5.2C).

### 5.2.3 Raf-BxB T481A activates NF-κB

The T481A mutation uncouples MEK1 activation from the active Raf kinase. The absence of ERK1/2 phosphorylation in Raf-BxB T481A transfected cells could be the result of a failure of Raf to interact with its effectors. Pearson *et al.* (2000) showed that the T481A mutant was still capable of activating NF-κB dependent transcription, suggesting that Raf effector pathways other than MEK were still activated. Extracting nuclear proteins from Raf transfected cells, NF-κB activity was measured by electro-mobility shift assay (EMSA) with a [$^{32}$P]-labelled NF-κB oligonucleotide containing NF-κB DNA binding sites. Binding reactions performed between the labelled NF-κB oligonucleotide and nuclear extracts from transfected cells were resolved on a 6% non-denaturing polyacrylamide gel. Binding of the nuclear extracts to labelled oligonucleotide (p65 and p50) was detected by

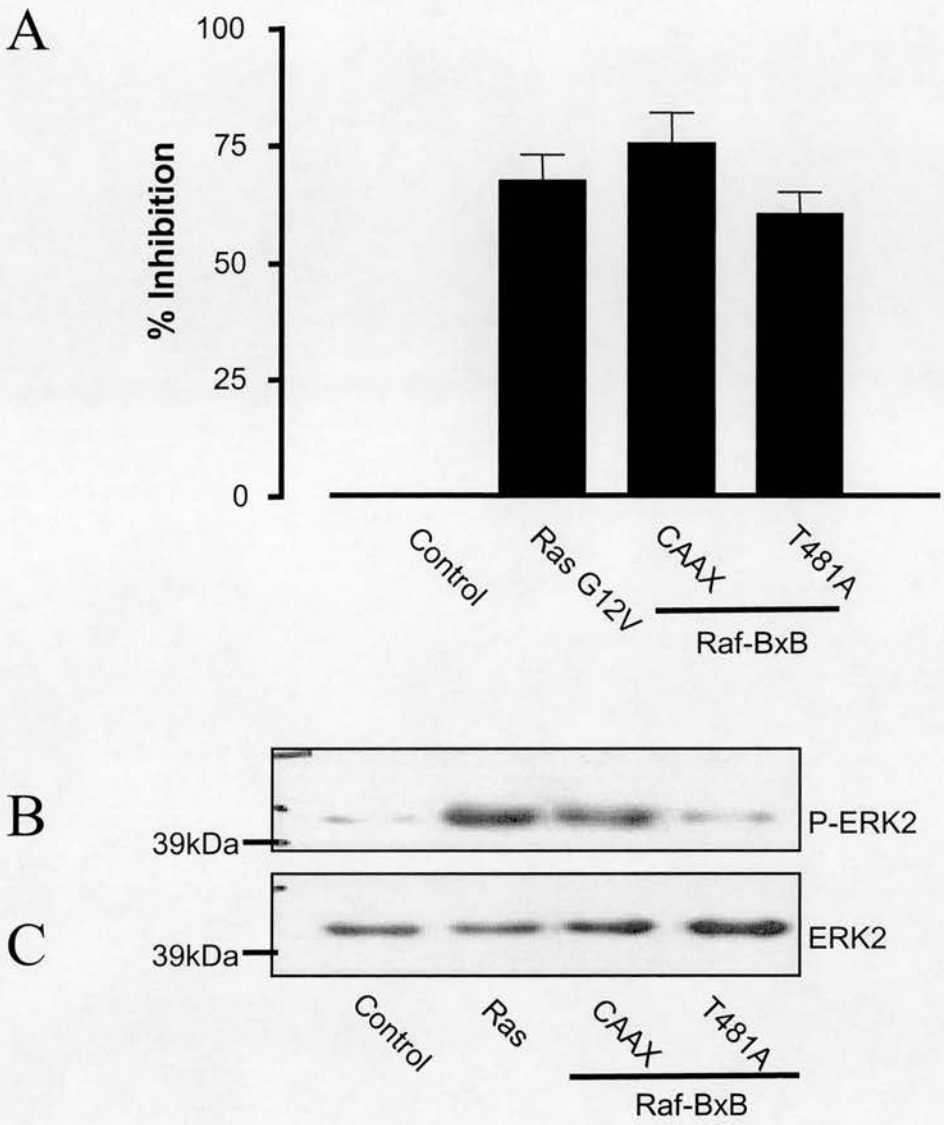


**Figure 5.2 Effect of Raf-BxB T481A on integrin affinity and ERK1/2 phosphorylation.**

(A) Integrin affinity was determined in Raf-BxB CAAX (1μg) and the T481A mutant (1μg) transfected cells. Ras G12V (1μg) was transfected as a positive control. Percentage inhibition was calculated by comparing the activation index in the presence of Raf DNA to that of the control vector. The results shown are the mean ± SEM of 3 independent experiments. (B) Cell lysates from transfected cells were probed with the phospho-specific ERK antibody and (C) the ERK2 antibody. The western blots are representative of 3 experiments.

autoradiography. Figure 5.3A shows that NF-κB binding was increased in nuclear lysates from Raf-BxB CAAX and T481A transfected cells compared to control vector transfected cells (representative autoradiograph of three experiments). Ras G12V transfected cells also displayed an increase in NF-κB activity.

Control EMSA reactions were performed (Figure 5.3B) to show that binding reactions were specific for NF-κB. Nuclear extracts from Raf-BxB T481A were incubated with either excess unlabelled NF-κB oligonucleotide or excess unlabelled SP-1 oligonucleotide which does not bind NF-κB. the NF-κB oligonucleotide eliminated NF-κB binding while the non-specific (SP-1) oligonucleotide had no effect on NF-κB binding to the NF-κB specific oligonucleotide. In addition a supershift reaction was performed. Nuclear extracts were incubated with the p65 antibody prior to the binding reaction. P65 is a subunit of NF-κB and a supershift indicates that nuclear extracts specifically contain the p65 subunit. The other band is thought to be the second subunit of NF-κB. Other unlabelled bands present on the gel are thought to be non-specific bands or are a result of protein breakdown.

Image quantification of phospho-images with ImageQuant software allowed a numerical estimate of NF-κB activity. NF-κB activity was increased 4.2 ± 1.1 fold over control vector transfected cells in Raf-BxB T481A transfected cells (Figure 5.4). A fold increase of 2.3 ± 0.9 and 3.0 ± 1.3 was observed for Raf-BxB CAAX and Ras G12V transfected cells respectively. These results indicate that the Raf-BxB T481A mutant is capable of activating NF-κB and possibly other signalling pathways in the absence of MEK activation.

## 5.3 Effect of constitutively active MEK1 on integrin affinity

Integrin suppression by Raf-BxB T481A suggests that suppression may occur in the absence of MEK activation. Utilising an active MEK1 mutant (MEK1-DD, S218D S222D), the requirement for MEK activation during Raf-mediated suppression was determined.

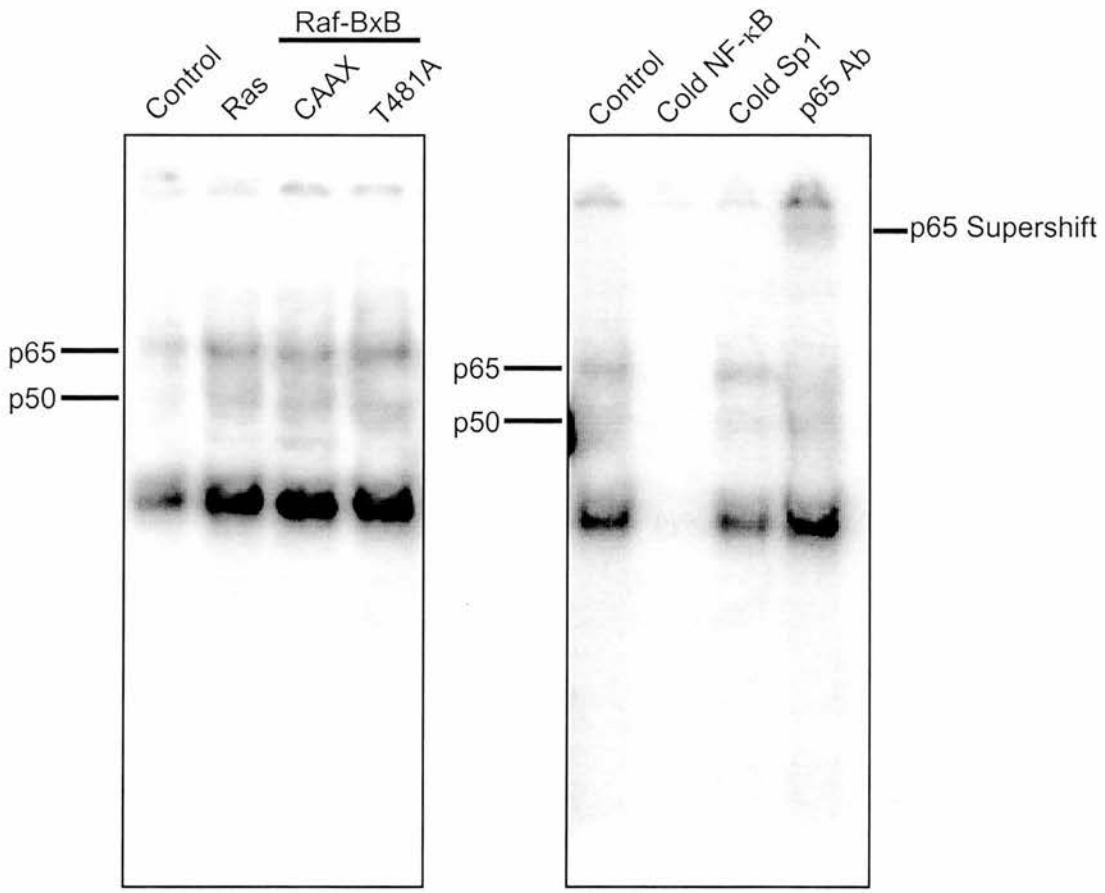


**Figure 5.3 NF-κB binding by Raf-BxB T481A.**

(A) NF-κB EMSA's were performed on nuclear extracts from Raf-BxB transfected cells (1μg DNA). (B) Control NF-κB binding reactions were performed on the nuclear extract from Raf-BxB T481A transfected cells. Binding reactions were performed in the presence of either excess cold NF-κB oligonucleotide, the non-specific SP1 oligonucleotide or the p65 antibody. The supershifted NF-κB complex is marked on the autoradiograph.

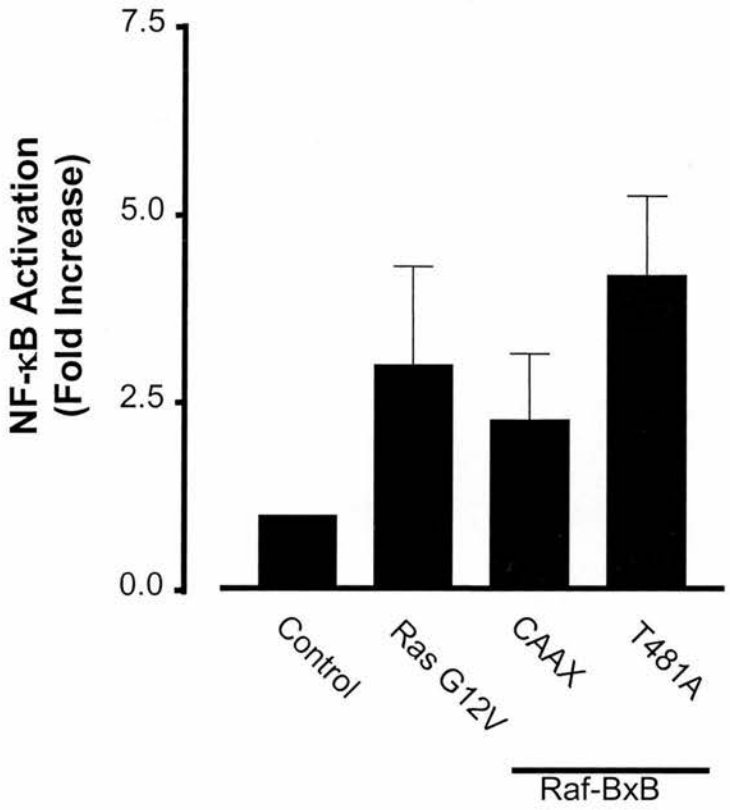


**Figure 5.4 NF-κB activity in Raf-BxB transfected cells.**

Autoradiographs of NF-κB activity of transfected cells (1μg) were subjected to the ImageQuant analysis software. Densitometry performed on the p65/p50 subunit bands are represented as the fold increase over control vector transfected cells. The results shown are the mean ± SEM of three independent experiments.

### 5.3.1 Active MEK leads to an increase in ERK1/2 phosphorylation

Expression of the Ha-tagged MEK1 in αβ-py cells (Figure 5.5A) revealed a single protein band at 45kDa, detected with the anti-Ha antibody; Ras G12V was expressed as a 23kDa protein. Probing lysates with the phospho-ERK specific antibody showed that expression of MEK1-DD resulted in a large increase in ERK1/2 phosphorylation (Figure 5.5B). Phosphorylation of ERK1/2 in Ras G12V transfected cells produced a more modest increase in phosphorylation compared to MEK1-DD. Total ERK levels were unaffected by expression of either Ras G12V or MEK1-DD (Figure 5.5C).

### 5.3.2 MEK1 DD expression can mediate integrin suppression

Figure 5.6 showed that expression of MEK1-DD in αβ-py cells caused integrin suppression (53.5 ± 5.2%), similar to that with Ras G12V (69.5 ± 3.8%). The involvement of MEK1 in integrin suppression is in contradiction to previous data obtained with Raf-BxB T481A and PD098059 experiments that indicate that integrin suppression is independent of MEK and ERK1/2 activation.

## 5.4 Effect of constitutively active ERK2 on integrin affinity

Integrin suppression by Raf-BxB T481A indicates that Raf-mediated suppression occurs via an ERK1/2-independent pathway. However, integrin suppression by MEK1-DD suggests that activation of ERK1/2 may contribute to integrin suppression or that overexpression of the MEK protein can lead to phosphorylation of other substrates that could mediate integrin suppression.

Expression of a constitutively active ERK construct in αβ-py cells would determine whether ERK activation is required for integrin affinity modulation. An ERK2-MEK1 fusion construct generated by Robinson *et al.* (1998) produced an active ERK2 protein capable of activating transcription factors Elk-1 and AP-1 and phosphorylation of myelin basic protein (MBP) (Robinson *et al.*, 1998).

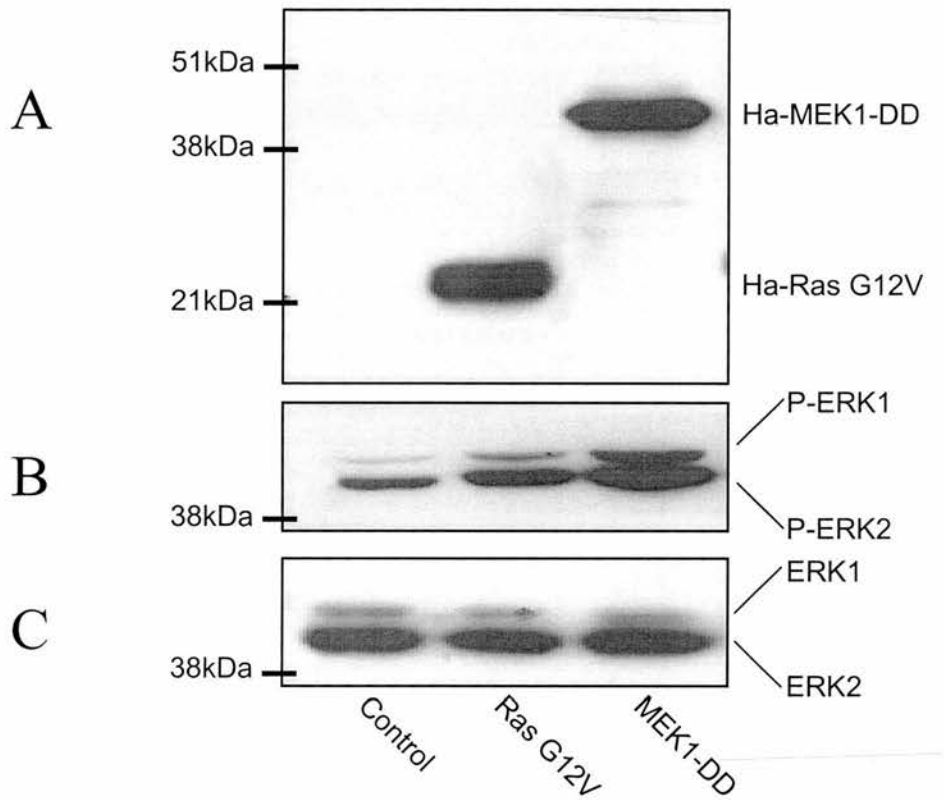


**Figure 5.5 Effect of MEK1-DD expression on ERK1/2 phosphorylation.**

Cell lysates from Ras G12V (1μg) and MEK1-DD (1μg) transfected cells were probed with the (A) anti-Ha antibody. Separate blots were also probed with (B) the phospho-specific ERK antibody and (C) the ERK2 antibody. The western blots shown are representative of 2 experiments.

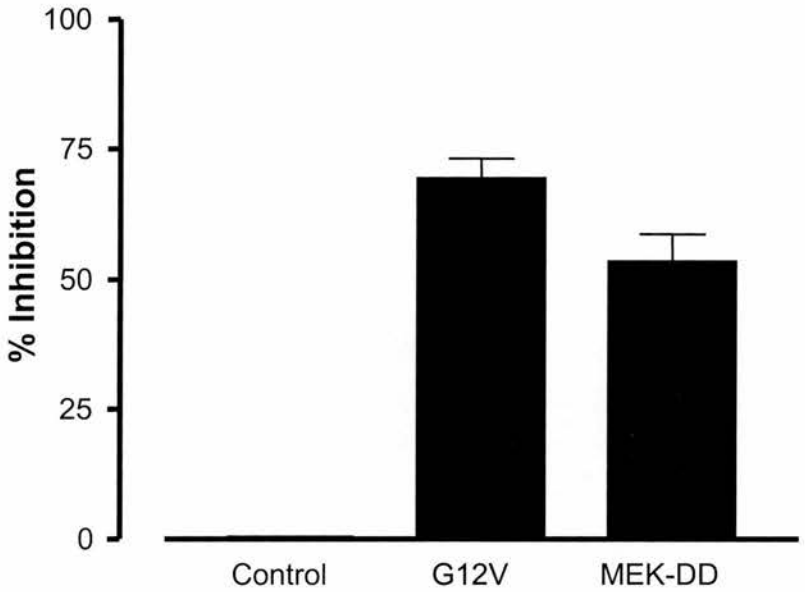


**Figure 5.6 Effect of MEK1-DD expression on integrin affinity.**

Integrin affinity was determined in MEK1-DD transfected cells (1μg). Ras G12V (1μg) was transfected as a positive control. Percentage inhibition was calculated by comparing the activation index in the presence of test DNA to that of the empty vector. The results shown are the mean ± SEM of 5 independent experiments.

### 5.4.1 Active ERK2-MEK1 phosphorylates MBP

Cells transfected with a construct encoding myc-tagged wildtype ERK2-MEK1 fusion protein revealed a protein of approximately 95 kDa on western blots probed with the anti-myc (9E10) antibody (Figure 5.7A). Increasing DNA transfected from 1μg to 3μg of DNA increased expression of the fusion protein. Mutation of $Lys^{52}$ to Arg within ERK2 produces a protein that is phosphorylated by the linked MEK1 but has no endogenous ERK2 kinase activity i.e. kinase dead (Robinson *et al.*, 1998). Transfection of this kinase-dead fusion protein also revealed a protein of 95 kDa, however, expression of this construct was greater than that of the wildtype construct. Increasing the quantity of wildtype ERK2-MEK1 DNA beyond 3μg failed to increase expression of the construct to levels equivalent to that of the kinase dead mutant.

ERK2 activity was assayed by immunoprecipitation of the myc-tagged ERK2-MEK1 fusion proteins with the anti-myc (9E10) antibody and MBP used as a substrate. Figure 5.7B shows myelin basic protein (MBP) phosphorylation represented as a fold increase above control pCMV5 vector, by the ERK2-MEK1 fusion proteins. Wildtype ERK2-MEK1 increased MBP phosphorylation by 2.6 ± 0.7 and 6.1 ± 1.6 fold with 1 and 3μg DNA respectively. Transfection of 3μg of wildtype ERK2-MEK1 DNA caused a significant increase ($P<0.05$) in MBP phosphorylation compared to control cells. Immunoprecipitation of the kinase-dead ERK2-MEK1 fusion protein failed to increase MBP phosphorylation over control vector levels (0.6 ± 0.2 for 1μg and 0.2 ± 0.2 fold for 3μg DNA) that were not significantly different ($P>0.05$). Reducing ERK2-MEK1 kinase-dead expression levels to that similar of the wildtype fusion proteins also failed to increase MBP phosphorylation (data not shown). This data indicates that the wildtype ERK2-MEK1 protein is catalytically active in αβ-py cells and acts as a constitutively active kinase within αβ-py cells.

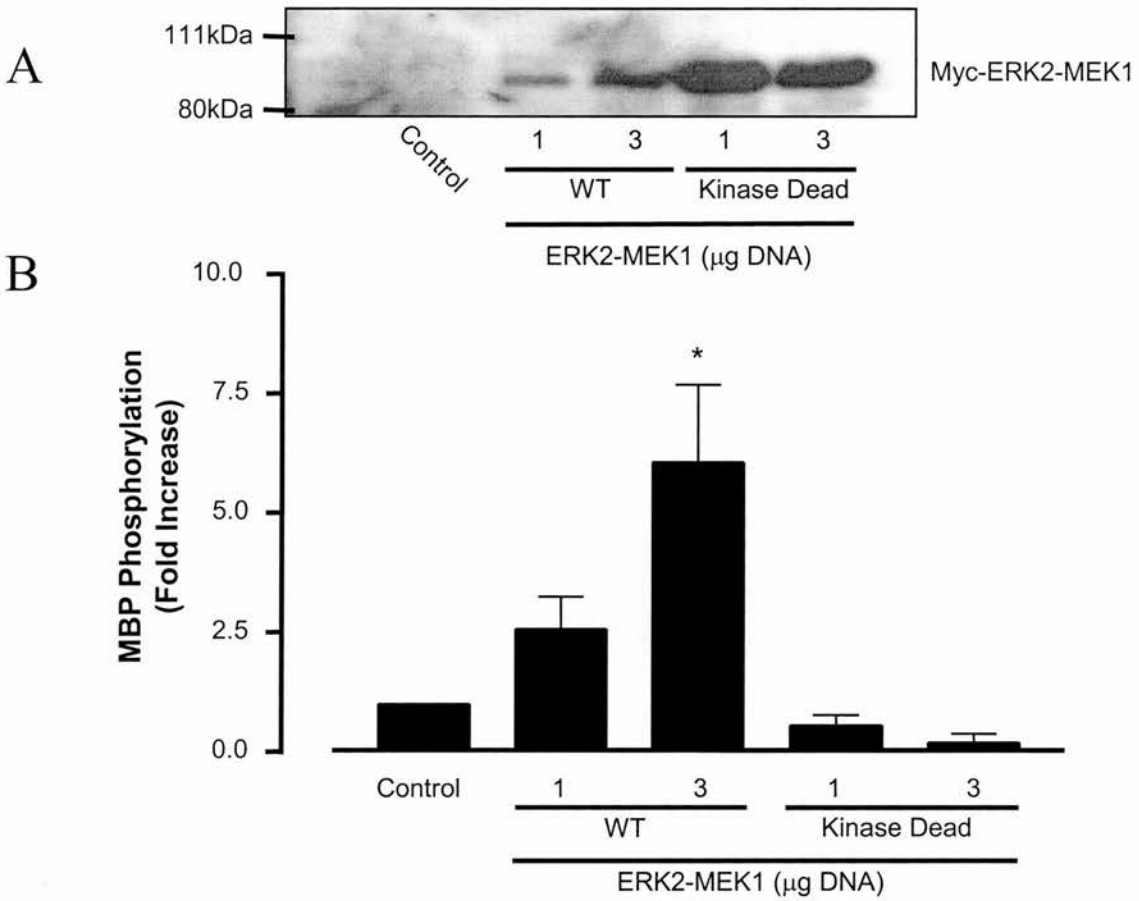


**Figure 5.7 Effect of expression of ERK2-MEK1 fusion proteins on MBP phosphorylation.**

(A) Cell lysates from ERK2-MEK1 transfected cells (1-3μg DNA) were probed with the anti-myc (9E10) antibody. The western blots shown are representative of 3 experiments. (B) Kinase activity of immuno-precipitated fusion proteins was assessed by MBP phosphorylation. The results, which are expressed as fold increase over control vector transfected cells (3μg), are the mean ± SEM of three independent experiments carried out in duplicate. Statistical analysis was performed by one-way ANOVA test, asterisk indicates significant difference ($P<0.05$) compared to control transfected cells.

### 5.4.2 Active ERK2-MEK1 does not suppress integrins

Expression of wildtype ERK2-MEK1 in αβ-py cells yields a constitutively active kinase capable of phosphorylating MBP. Integrin affinity was assessed in cells expressing either active ERK2 or the kinase-dead mutant. Transfection of the pCMV5 empty vector caused a small suppressive effect in αβ-py cells (15.7 ± 4.0%) (Figure 5.8). Expression of wildtype ERK2-MEK1 with 1 or 3μg of DNA in αβ-py cells did not suppress the chimeric integrin beyond that of the pCMV5 control vector (10 ± 3.8% and 0.02 ± 7.1% respectively). The kinase-dead ERK2-MEK1 fusion protein was also unable to suppress the chimeric integrin (4.7 ± 10.2% and –2.5 ± 10.1% respectively for 1 and 3μg of DNA). Compared to either control or empty vector transfected cells, expression of ERK2-MEK1 fusion proteins did not cause any significant difference ($P>0.05$) in percentage inhibition.

This data indicates that while expression of a constitutively active ERK2 in αβ-py cells is capable of phosphorylation of MBP, no effect on integrin affinity was observed.

## 5.5 Effect of MKP-1 on Raf-BxB T481A

Integrin suppression by Ras G12V was reversed by co-transfection of MKP-1 (dephosphorylates Thr/Tyr residues in MAP kinase proteins) (Figure 3.11). A similar reversal of integrin suppression was also observed with the Ras (G12V, T35S) mutant (Figure 4.6). This data suggests that integrin suppression by the Raf effector arm downstream of Ras may be sensitive to MKP-1 expression. Raf-BxB T481A suppresses integrins in the absence of ERK1/2 phosphorylation. Co-expression of MKP-1 with Raf-BxB T481A was performed to determine whether an ERK-like activity other than ERK1/2 was responsible for Raf-BxB T481A suppression.

### 5.5.1 MKP-1 prevents ERK1/2 phosphorylation by Raf

Cell lysates from co-transfections with Raf and MKP-1 were subjected to western analysis for expression of the Raf and MKP-1 constructs. Ha-tagged Raf-BxB

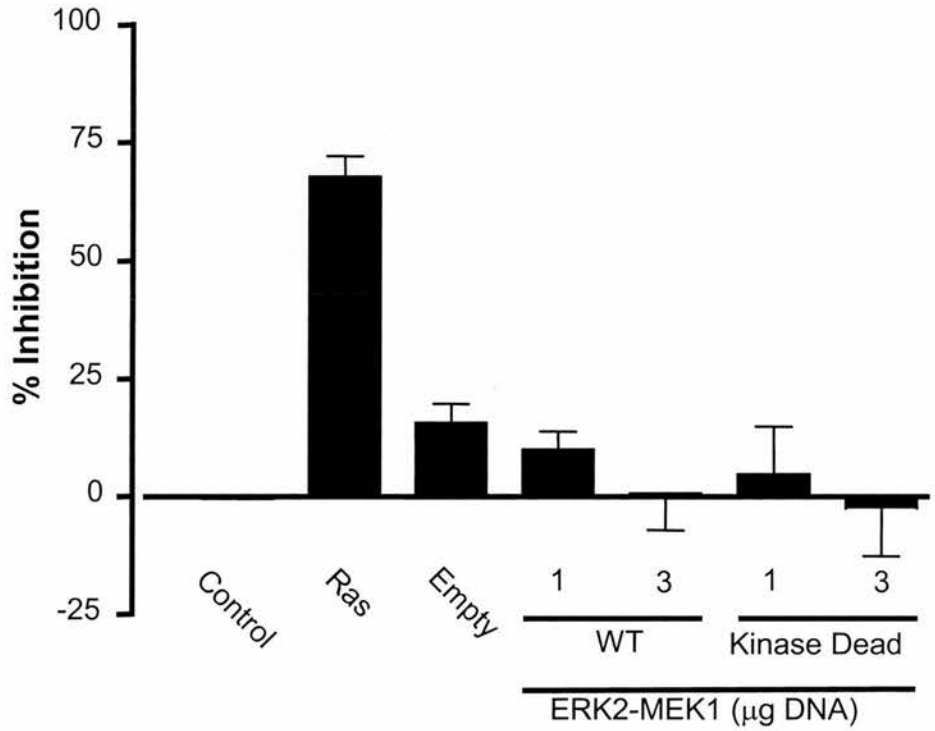


**Figure 5.8 Effect of ERK2-MEK1 fusion proteins on integrin affinity.**

Integrin affinity was determined in cells transfected with either the wildtype or the kinase dead ERK2-MEK1 fusion proteins. Ras G12V (1μg) was transfected as a positive control, the ERK2-MEK1 empty vector (3μg) was transfected as a control for the ERK2-MEK1 constructs. Percentage inhibition was calculated by comparing the activation index in the presence of ERK2-MEK1 DNA to that of the control vector (3μg). The results shown are the mean ± SEM of 3-5 independent experiments. Statistical analysis was performed by one-way ANOVA test.

CAAX was expressed as a single band at 42-44kDa and expression was unaffected by co-transfection of MKP-1 (Figure 5.9A). Expression of Raf-BxB T481A was detected with the anti-Raf antibody and as previously described a band at 37kDa was observed (Figure 5.9B). Co-transfection of MKP-1 did not affect expression of Raf-BxB T481A. The myc-tagged MKP-1 was probed with the anti-myc (A14) antibody, the anti-myc (9E10) antibody previously used for other myc-tagged proteins failed to recognise either MKP-1 or Raf-BxB T481A proteins (Figure 5.9C). MKP-1 was expressed as a 39kDa protein, a small reduction in MKP-1 expression was observed in Raf-BxB T481A co-transfections compared to expression in control vector or Raf-BxB CAAX co-transfections. The anti-myc (A14) antibody also detected the myc-tagged Raf-BxB T481A protein at 37kDa.

Cell lysates from co-transfections with Raf and MKP-1 were probed with the phospho-specific ERK antibody (Figure 5.9D). As previously observed, Raf-BxB CAAX expression produced an increase in ERK2 phosphorylation compared to control lysates. Raf-BxB T481A expression did not increase ERK2 phosphorylation above control vector transfected cells. Co-expression of MKP-1 in all three transfections prevented ERK2 phosphorylation. Low level phosphorylation was occasionally observed in MKP-1 co-transfections upon long film exposure times. ERK2 expression levels were unaffected by Raf or MKP-1 expression in αβ-py cells compared to control cells (Figure 5.9E).

### 5.5.2 MKP-1 expression inhibits Raf-mediated integrin suppression

MKP-1 co-expression prevented the increase in ERK2 phosphorylation by Raf-BxB CAAX and basal ERK2 phosphorylation by Raf-BxB T481A. The ability of MKP-1 to inhibit integrin suppression by Ras G12V and the Ras (G12V, T35S) mutant indicated that MKP-1 expression might also inhibit integrin suppression by Raf. Expression of Raf-BxB CAAX or Raf-BxB T481A both increased integrin suppression compared to that observed with Ras G12V (Figure 5.10). Co-transfection of MKP-1 with empty vector produced a small increase in PAC1 binding (-8 ± 15.1% inhibition), this was previously observed in Ras G12V-MKP-1 co-

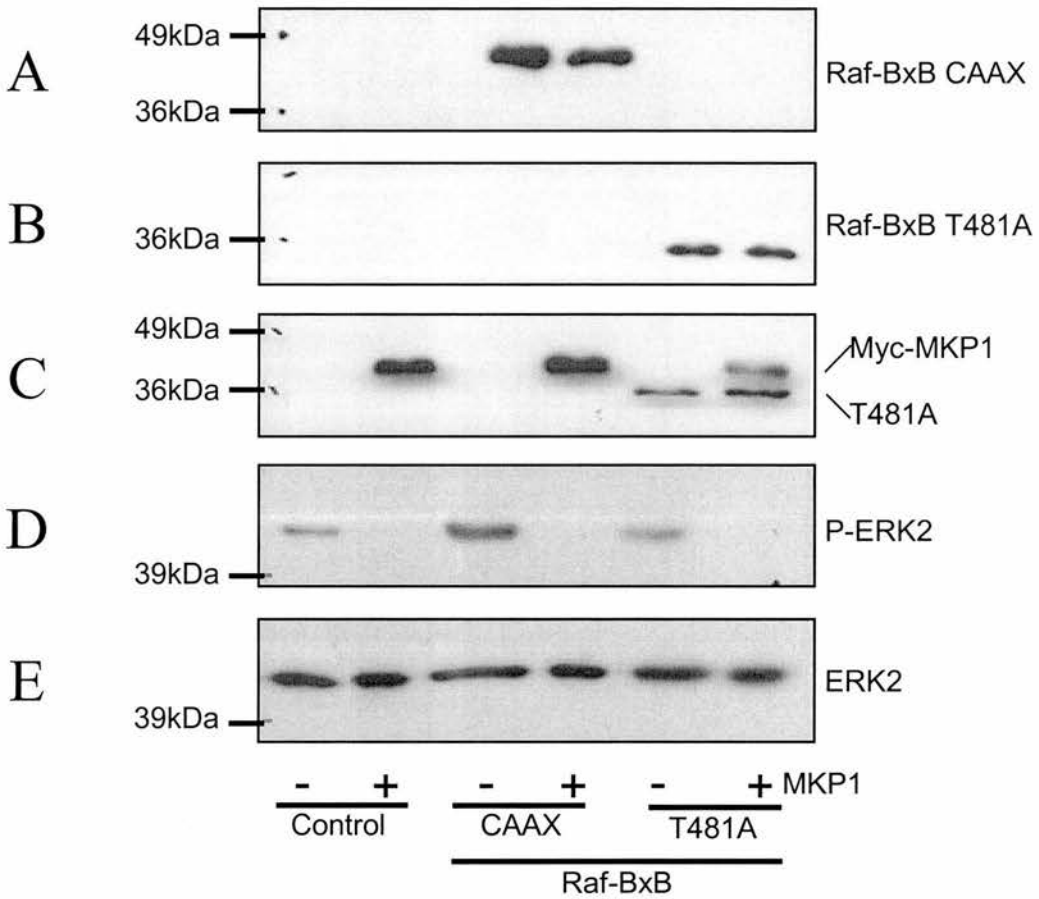


**Figure 5.9 Effect of MKP1 co-expression on Raf-BxB mediated ERK1/2 phosphorylation.**

Lysates from cells co-transfected with Raf-BxB (1μg) and MKP-1/empty vector (1μg) were probed with (A) anti-Ha antibody, (B) Raf antibody (E10) and (C) the anti-myc (A14) antibody. Lysates were also probed with (D) the phospho-specific ERK antibody and (E) the ERK2 antibody. The western blots shown are representative of 3 experiments.

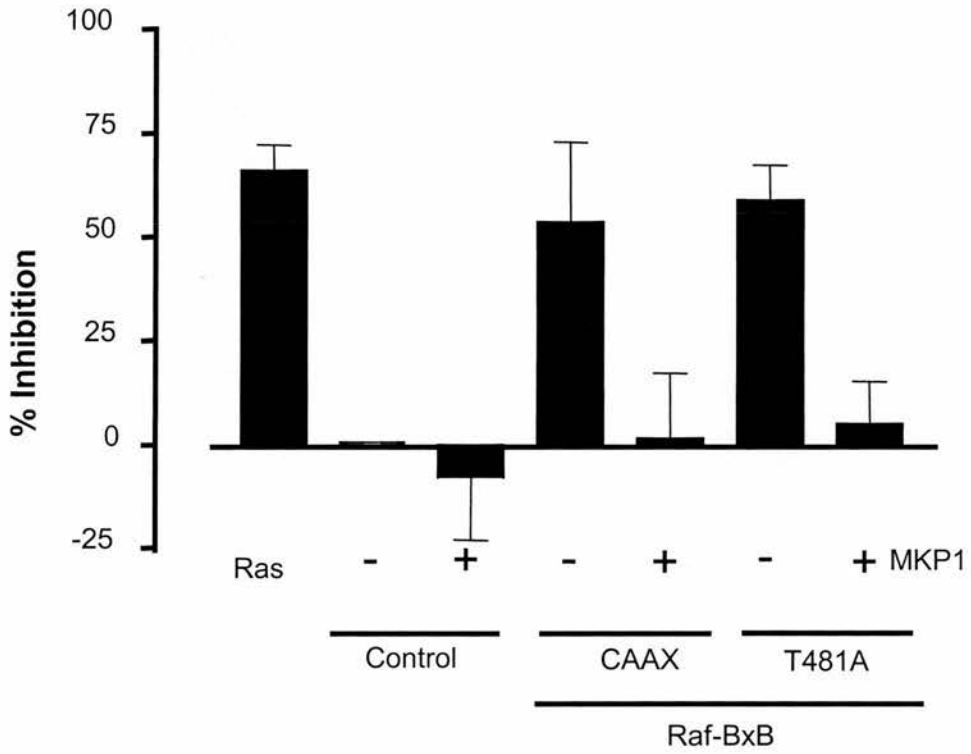


**Figure 5.10 Effect of MKP1 expression on integrin affinity in Raf-BxB co-transfected cells.**

Integrin affinity was determined in Raf-BxB transfected cells (CAAX or T481A, 1μg) co-transfected with either MKP1 or control vector (1μg). Percentage inhibition was calculated by comparing the activation index in the presence of test DNA to that of the control vector in the absence of MKP-1. The results shown are the mean ± SEM of 3 independent experiments.

transfections (Figure 3.11). Expression of MKP-1 with Raf-BxB CAAX (1.6 ± 15.5% inhibition) produced a 97% reduction in integrin suppression compared with Raf-BxB CAAX alone (53.6 ± 19.3%). Co-transfection of MKP-1 with Raf-BxB T481A (58.9 ± 8.4%) produced a similar reduction in integrin suppression. A 91 % reduction was observed in MKP-1 and Raf-BxB T481A co-transfectants (5.2 ± 10.0%).

The ability of MKP-1 to reverse integrin suppression by both Raf constructs suggests that ERK1/2-independent integrin suppression by Raf-BxB T481A may require a MKP-1-sensitive ERK-like kinase activity.

## 5.6 Effect of R-Ras G38V on integrin suppression by Raf T481A

The active R-Ras small GTP binding protein (R-Ras G38V) has been shown to reverse integrin suppression caused by Ras G12V, Raf-CAAX and Raf-BxB (Sethi *et al.*, 1999). It was shown that R-Ras G38V can also reverse integrin suppression by the Ras effector mutants (Figure 4.11). We hypothesised that integrin suppression by Raf-BxB T481A should also be reversed by R-Ras G38V.

### 5.6.1 Effect of R-Ras G38V on ERK activation

Lysates from cells transfected with Raf mutants and R-Ras G38V were analysed for expression of the transfected constructs. Figure 5.11A shows that the Ha-tagged Raf-BxB CAAX construct was expressed as previously observed and that co-transfection of R-Ras G38V did not affect expression. Expression of Raf-BxB T481A was also unaffected by R-Ras G38V expression (Figure 5.11B). The myc-tagged R-Ras G38V was expressed as a 25kDa protein and was expressed at similar levels in all co-transfections performed (Figure 5.11C).

Cell lysates from co-transfections between Raf and R-Ras G38V were probed with the phospho-specific ERK antibody (Figure 5.11D). Raf-BxB CAAX expression produced an increase in ERK2 phosphorylation compared to control lysates while

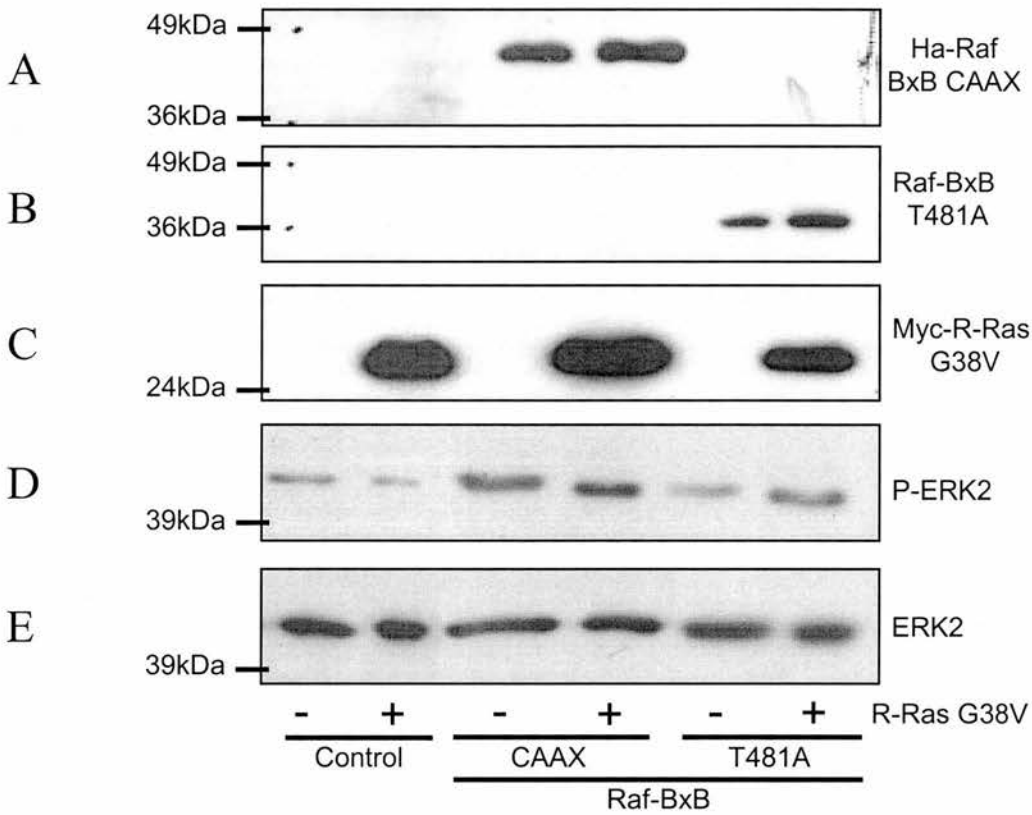


**Figure 5.11 Effect of R-Ras G38V expression on Raf-BxB mediated ERK1/2 phosphorylation.**

Cells transfected with either Raf-BxB CAAX (1μg) or Raf-BxB T481A (1μg) were co-transfected with R-Ras G38V or empty vector (1μg). Lysates were probed with (A) anti-Ha antibody, (B) anti-Raf E10 antibody or (C) anti-myc (9E10) antibody. Lysates were also probed with the (D) phospho-specific ERK antibody and (E) the ERK2 antibody. The western blots shown are representative of 3 experiments.

Raf-BxB T481A expression did not increase ERK2 phosphorylation above control vector transfected cells. Co-expression of R-Ras G38V with either control vector or Raf-BxB CAAX produced no detectable change in ERK2 phosphorylation. A slight increase in ERK2 phosphorylation was observed in co-transfections between Raf-BxB T481A and R-Ras G38V. The phosphorylation of ERK2 in these transfections was always below that observed with Raf-BxB CAAX. ERK2 expression levels were unaffected by Raf or MKP-1 co-expression in αβ-py cells compared to control cells (Figure 5.11E).

### 5.6.2 R-Ras G38V reverses Raf-mediated integrin suppression

Expression of either Raf-BxB CAAX (53.6 ± 19.3%) or Raf-BxB T481A (58.9 ± 8.4%) in αβ-py cells both suppressed integrins (Figure 5.12). This suppression by the Raf constructs was similar to that observed with Ras G12V. Co-transfection of R-Ras G38V expression with Ras G12V empty vector produced a small activation of the integrin (-7 ± 10.0%) compared to control vector alone. Co-expression of Raf-BxB CAAX with R-Ras G38V reduced integrin suppression from 53.6 ± 19.3% to 2 ± 11.1%, a reduction of 96 % in integrin suppression. R-Ras G38V was also capable of reversing integrin suppression by Raf-BxB T481A. Integrin suppression fell from 58.9 ± 8.4 to 6.1 ± 9.1%, a reduction of 90% in integrin suppression.

These results suggest that the ERK1/2 independent integrin suppression pathway of Raf-BxB T481A is still sensitive to R-Ras G38V expression.

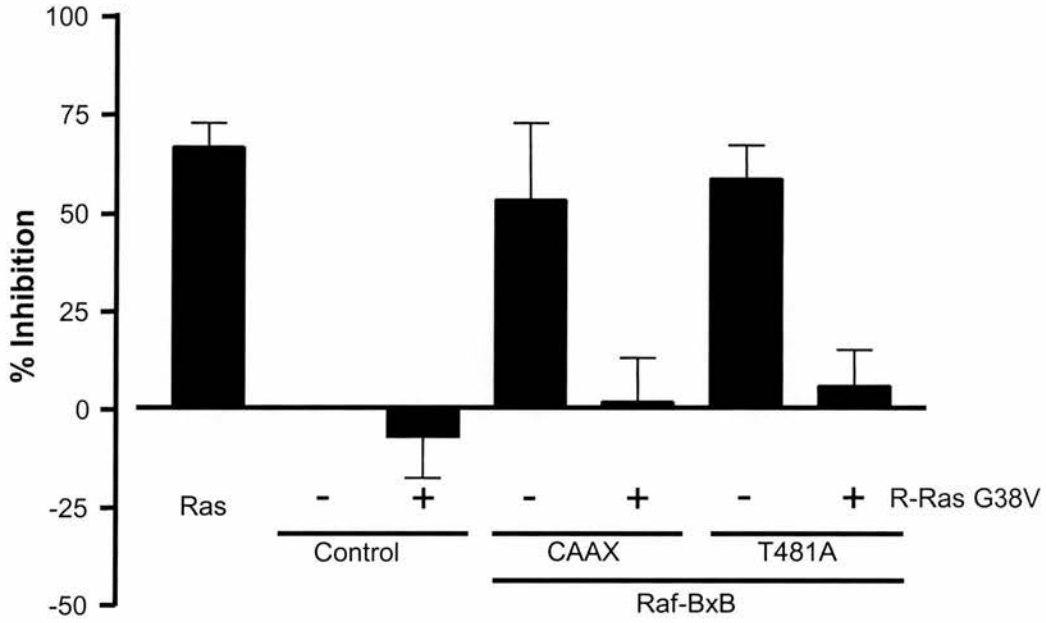


**Figure 5.12 Effect of R-Ras G38V expression on integrin affinity in Raf-BxB transfected cells.**

R-Ras G38V (1μg) was co-transfected together with Raf-BxB CAAX and the T481A mutant (1μg) and integrin affinity was determined in these cells. Percentage inhibition was calculated by comparing the activation index in the presence of test DNA to that of the control vector in the absence of R-Ras. The results shown are the mean ± SEM of 3 independent experiments.

## 5.7 Discussion

The results presented in this chapter show that a Raf-BxB mutant unable to induce ERK1/2 activation was capable of integrin suppression. This suppression was not dependent on ERK2 activation but was sensitive to MKP-1 co-transfection. Expression of a constitutively active R-Ras construct was also able to reverse the suppressive effects of Raf-BxB T481A and Raf-BxB CAAX.

Pearson *et al.* (2000) have shown that the Raf-BxB T481A mutant does not increase ERK1/2 phosphorylation. The absence of ERK1/2 phosphorylation in transfected αβ-py cells with Raf-BxB T481A was in correlation with observations in both HEK293 and PC12 cells (Pearson *et al.*, 2000). It was shown that the T481A mutant retained its ability to activate NF-κB signalling, measured by an increase in NF-κB translocation to the nucleus. The ability of Raf-BxB T481A to mediate NF-κB dependent transcription agrees with a previous report that inhibition of the Raf-ERK1/2 cascade with PD098059 or a dominant negative MEK did not affect Raf stimulated NF-κB dependent transcription (Baumann *et al.*, 2000). NF-κB dependent transcription is not believed to be responsible for Raf-mediated integrin suppression. Hughes *et al.* (1997) have shown that an estradiol-activated Raf protein was able to mediate a loss in PAC1 binding in the presence of either cyclohexamide or actinomycin D (Hughes *et al.*, 1997). This suggests that Raf-mediated suppression occurs in the absence of de novo protein synthesis and mRNA transcription.

Ramos *et al.* (1998) have previously reported that expression of an active MEK2 construct could suppress integrins (Ramos *et al.*, 1998). The ability of a constitutively active MEK1 construct to suppress integrins in αβ-py cells contradicts the previous data with Raf-BxB T481A and PD098059 experiments which have indicated that MEK and ERK1/2 were not required for integrin suppression. Lenormand *et al.* (1996) have shown that an active MEK1 construct led to an increase in p70 S6K activity in CCL39 cells, similar to that observed with an active Raf construct. However, Raf-mediated activation of p70 S6K activity was not reduced by co-expression of a dominant negative ERK1 or MKP-1. The authors

suggested that stimulation of p70 S6K activity by Raf was independent of ERK1/2 activation and that the effect of active MEK was due to the triggering of an autocrine loop subsequent to the persistent activation of the ERK1/2 cascade (Lenormand *et al.*, 1996). The large increase in ERK1/2 phosphorylation observed in MEK1-DD transfected cells (Figure 5.5) is consistent with previous use of active MEK constructs, Mansour *et al.* (1994) observed an 85-fold increase in ERK2 phosphorylation by an active MEK1 construct (Mansour *et al.*, 1994). MEK1 is highly specific for ERK1/2 and has not been shown to phosphorylate any other member of the MAP kinase superfamily (Dhanasekaran and Premkumar, 1998). Expression of an active MEK1 in HEK 293 and COS cells resulted in the hyperphosphorylation of Raf and an increase in Raf kinase activity (Zimmermann *et al.*, 1997). The ability of MEK1 to stimulate Raf activity in cells could explain the ability of MEK1-DD to suppress integrins. Preliminary experiments in αβ-py cells show that MEK1-DD can indeed stimulate endogenous Raf activity. The kinase responsible for Raf phosphorylation downstream of MEK remains to be identified, although phosphorylation was shown to be independent of Src activity (Zimmermann *et al.*, 1997).

The wildtype ERK2-MEK1 fusion protein expressed in HEK 293 cells caused an increase in MBP phosphorylation and induced Elk-1 dependent transcription (Robinson *et al.*, 1998). This ability of wildtype ERK2-MEK1 to phosphorylate MBP was reproduced in αβ-py cells, suggesting that this construct is constitutively active in αβ-py transfected cells. Endogenous ERK, upon activation by MEK translocates to the nucleus (Chen *et al.*, 1992; Lenormand *et al.*, 1993; Sanghera *et al.*, 1992) where it is known to phosphorylate several transcription factors (Su and Karin, 1996). The majority of wildtype ERK2-MEK1 however remains in the cytoplasm due to a leucine-rich nuclear export sequence in MEK1, mutation of these residues to alanine increases localisation of this protein into the nucleus (Robinson *et al.*, 1998). This nuclear localised ERK2-MEK1 was not used in these experiments as it is proposed that Ras G12V-mediated integrin suppression does not require de novo transcription or translation and that cytoplasmic signalling downstream of Ras is

sufficient for integrin suppression (Hughes *et al.*, 1997). Cytoplasmic ERK1/2 can phosphorylate cytoplasmic substrates; $p90^{RSK}$ activation by ERK (Sturgill *et al.*, 1988) allows its translocation into the nucleus where it too stimulates several transcription factors (Chen *et al.*, 1992; Chen *et al.*, 1993). Cytoplasmic phospholipase A2 (cPLA2) (Lin *et al.*, 1993; Nemenoff *et al.*, 1993) and myosin light chain kinase (MLCK) (Morrison *et al.*, 1996) are also activated by ERK1/2 phosphorylation. Both cPLA2 and MLCK are potential candidates as effectors modulating integrin affinity. Production of arachidonic acid by cPLA2 (Kramer and Sharp, 1997) is required for $\alpha_{IIb}\beta_3$ activation in platelets (Shattil and Brass, 1987), MLCK modulates actin-myosin contraction (Kamm and Stull, 2001) and changes within the cytoskeleton may affect integrin affinity. Cytochalasin D, an inhibitor of actin polymerisation, produced a modest increase in $\alpha_{IIb}\beta_3$ affinity in platelets (Bennett *et al.*, 1999).

The inability of the constitutively active ERK2 to mediate integrin suppression suggests that ERK1/2 and its effectors, including cytosolic phospholipase A2 ($cPLA_2$), myosin light chain kinase (MLCK) and $p90^{RSK}$ are probably not involved in integrin affinity modulation (Klemke *et al.*, 1997; Milella *et al.*, 1997; Sturgill *et al.*, 1988). While wildtype ERK2-MEK1 is sufficient for MBP phosphorylation (Figure 5.7) and increase transcription (Robinson *et al.*, 1998), the presence of the fused MEK1 protein may hinder interactions of ERK2 with its effectors. Wildtype ERK2-MEK can phosphorylate $p90^{RSK}$, although phosphorylation of cPLA2 and MLCK has not been shown (Robinson *et al.*, 1998). As such, a role for cPLA2 and MLCK activation cannot be ruled out until inhibitor studies are performed e.g. MAFP (cPLA2 inhibitor) (Lio *et al.*, 1996) and ML-9 (MLCK inhibitor) (Saitoh *et al.*, 1986). However, the inability of active ERK2 to suppress integrins does fit with the premise that Ras and Raf-mediated suppression is independent of ERK1/2 activation.

A described in chapter 3, MKP-1 is a broad spectrum phosphatase, that is known to act on ERK1/2, JNK and p38 MAP kinase (Chu *et al.*, 1996). The ability of MKP-1 to reverse integrin suppression by Ras-BxB T481A (Figure 5.10) suggests that a member of the MAP kinase superfamily can mediate integrin suppression. The data

within this thesis indicates that this MAP kinase is not ERK1 or ERK2. Co-expression of MKP-3 (Pyst1) and Raf-BxB T481A produced similar results to that of MKP-1 (preliminary data). MKP-3 preferentially dephosphorylates MAP kinases with the TEY phosphorylation motif present in ERK1/2, with very low activity toward JNK (TPY) and p38 MAP kinase (TGY) (Groom *et al.*, 1996; Muda *et al.*, 1996). The increased specificity of MKP-3 indicates that a MAP kinase with a TEY motif i.e. an ERK family member is downstream of the Raf effector pathway that mediated integrin suppression. Possible candidate ERKs includes ERK5 and p97 ERK5-related protein.

ERK5 a protein approximately twice the size of the other ERK proteins (Lee *et al.*, 1995b; Zhou *et al.*, 1995) has shown kinase activity in response to activated Ras and Raf (English *et al.*, 1999). MEK5 the upstream activator of ERK5 can associate with Raf and activated Raf to a greater extent, however direct activation of MEK5 by Raf has not been shown (English *et al.*, 1995). Both MKP-1 and MKP-3 are able to dephosphorylate ERK5. However, the ability of PD098059 and U0126 to inhibit ERK5 phosphorylation by acting upon MEK5 argues against a possible role of ERK5 in Raf-mediated integrin suppression (Kamakura *et al.*, 1999).

Janulis *et al.* (2001) have recently identified a novel p97 ERK5-related protein by affinity purification. This protein associates with and was activated by Raf, but the MEK inhibitors PD098059 and U0126 did not inhibit activation of p97 (Janulis *et al.*, 2001). Cross-reaction of phospho-specific ERK5 antibodies with phosphorylated p97 suggests that the activation loop TEY motif may be present in p97 and therefore susceptible to dephosphorylation by MKP-1 and MKP-3 action. Of the remaining identified ERK proteins, none have been reported to interact with Raf.

A constitutively active form of R-Ras has been shown to reverse Ras G12V-mediated integrin suppression (Sethi *et al.*, 1999) and that of the Ras G12V effector mutants (Figure 4.11). R-Ras G38V can also reverse integrin suppression by Raf-BxB CAAX and Raf-BxB T481A (Figure 5.12). The mechanism of R-Ras reversal is currently unclear; Sethi *et al.* (1999) have proposed that R-Ras signals may act at a point downstream of ERK1/2. The ability of R-Ras G38V to reverse Raf-BxB

T481A-mediated suppression suggests that the novel Raf effector pathway is also sensitive to R-Ras signalling.

GTP loaded R-Ras can bind to Raf-1, however Raf activity is not increased by R-Ras (Marte *et al.*, 1997). This is reflected in the inability of R-Ras G38V to affect ERK1/2 phosphorylation in transfected cells (Sethi *et al.*, 1999) and (Figure 5.11). As the two Raf constructs used here lack the N-terminal Ras binding domains and thus cannot bind to R-Ras. Reversal of Raf-mediated integrin suppression therefore cannot be attributed to R-Ras sequestering Raf away from its effectors. Until effectors of R-Ras and Raf-BxB T481A are identified the mechanism by integrin suppression is reversed remains undetermined.

In summary it has been shown that integrin suppression by a Raf-BxB mutant can occur in the absence of ERK1/2 activation. Suppression is sensitive to co-expression of MKP-1 but does not occur in the presence of a constitutively active ERK2. Signalling by a novel Raf effector other than MEK may be responsible in activating an ERK-like kinase that mediates integrin suppression by Raf-BxB T481A.

# RESULTS: CHAPTER 6

# Search for Novel Integrin Affinity Modulators

## 6.1 Introduction

Ras-mediated integrin suppression appears to utilise several effector pathways. The precise association between effectors and integrins is currently unclear. Several proteins have been identified that can bind integrin cytoplasmic tails (reviewed in Liu 2000), only cytohesin1 however, has been shown to link $\beta_2$ integrin affinity and the Ras effector, PI3-kinase (Nagel *et al.*, 1998).

Model organisms have often been used to study complex biological processes. The power of *Drosophila* genetics has allowed rapid identification of loci associated with integrin-mediated adhesion. Currently, 5$\alpha$ and 2$\beta$ subunits have been identified in *Drosophila*; mutations in which reveal that integrins are required for both embryonic and larval development. Several phenotypes are observed with integrin mutations, including defects in muscle contraction and attachment to the epidermis, gut morphogenesis and epithelial cell layer adhesion (reviewed in Brown 2000).

Deletion of the $\alpha_{IIb}$ cytoplasmic tail generates a constitutively active integrin capable of binding PAC1 in CHO cells (O'Toole *et al.*, 1991). A similar deletion of the cytoplasmic domain of the $\alpha PS_2$ subunit produced abnormal and delocalised muscle attachments. Muscle attachment to the epidermis is normally restricted to the muscle end and mediated by the $\alpha_{PS2}\beta_{PS}$ integrin. Integrin activation, (normally restricted to the muscle tip) is hypothesised to mediate these ectopic attachments. Modulation of *drosophila* integrin affinity may therefore play an important role during development (Martin-Bermudo *et al.*, 1998).

The wing blister phenotype is associated with a loss of integrin-mediated adhesion between merging epidermal layers. Genetic screens performed by Prout *et al.* (1997) and Walsh *et al.* (1998) have identified several novel loci required for integrin

adhesion (Prout *et al.*, 1997; Walsh and Brown, 1998). In collaboration with Dr. N. Brown (Cambridge, UK) we have obtained genes encoding *cassowary* (*Cass*) and *Auk*. Neither gene altered integrin expression level and sequence information suggests that they are both cytoplasmic proteins. It can be speculated that these proteins may have a role in affinity modulation.

Expression cloning screening strategies have been successful in identifying proteins that modulate integrin affinity. CD98 can reverse trans-dominant suppression by $\beta_1$ tails while PEA-15 can reverse Ras G12V-mediated integrin suppression (Fenczik *et al.*, 1997; Ramos *et al.*, 1998). Small cell lung cancer (SCLC) is a highly metastatic tumour and *in vitro*, cells grow as free floating aggregates. Suppression of integrin affinity may explain the *in vitro* growth characteristics of these cells. To date, oncogenic Ras mutations have not been identified in SCLC tumours and therefore a novel effector may mediate integrin suppression in these cells (Suzuki *et al.*, 1990).

The aim of this part of the study was to use alternative methods to identify proteins involved in integrin affinity modulation.

## 6.2 Cloning of *Drosophila* genes into a mammalian expression vector

The *drosophila* genes encoding Cassowary (Cass) and Auk have been implicated in integrin-mediated adhesion between the dorsal and ventral layers of the adult wing (Walsh and Brown, 1998). To determine whether they play a role in modulating affinity of drosophila integrins, we sought to express these genes in αβ-py cells.

### 6.2.1 High fidelity PCR of Cass and Auk

Both drosophila genes provided by Dr. N. Brown (Cambridge, UK) were cloned into vectors unsuitable for mammalian cell expression. Cass was cloned in the Bluescript cloning vector and Auk in the pWRhpA drosophila vector. In order to clone these genes into a suitable mammalian expression vector, high fidelity PCR was performed using Stratagene's Pfu Turbo DNA polymerase. Primers were designed to incorporate unique restriction sites in order to facilitate subsequent sub-cloning steps. PCR cycle conditions were optimised as described in Materials and Methods and PCR products were amplified as shown in Figure 6.1. The Cass gene was amplified as a 1.6kb product (Figure 6.1A) and the Auk gene as a 2.6kb product (Figure 6.1B). No PCR products were observed in reactions performed in the absence of template DNA in both Cass and Auk PCR reactions.

### 6.2.2 Cloning of genes into the pCMV-Tag3B expression vector

Ethanol precipitated PCR products were subjected to poly-A tail reactions and cloned into a pGEMT vector using the Promega Rapid ligation kit. Ligations were transformed into competent DH5α E.coli and screened by blue/white selection. Figure 6.2 shows restriction digests of ligations with appropriate restriction enzymes of amplified DNA from white colonies on the ligation plates. Clones that contained inserts of the correct size were subjected to automated fluorescence sequencing. DNA sequencing showed that Cass clone 14 and Auk clone 13 contained inserts with identical DNA sequence to that of template DNA.

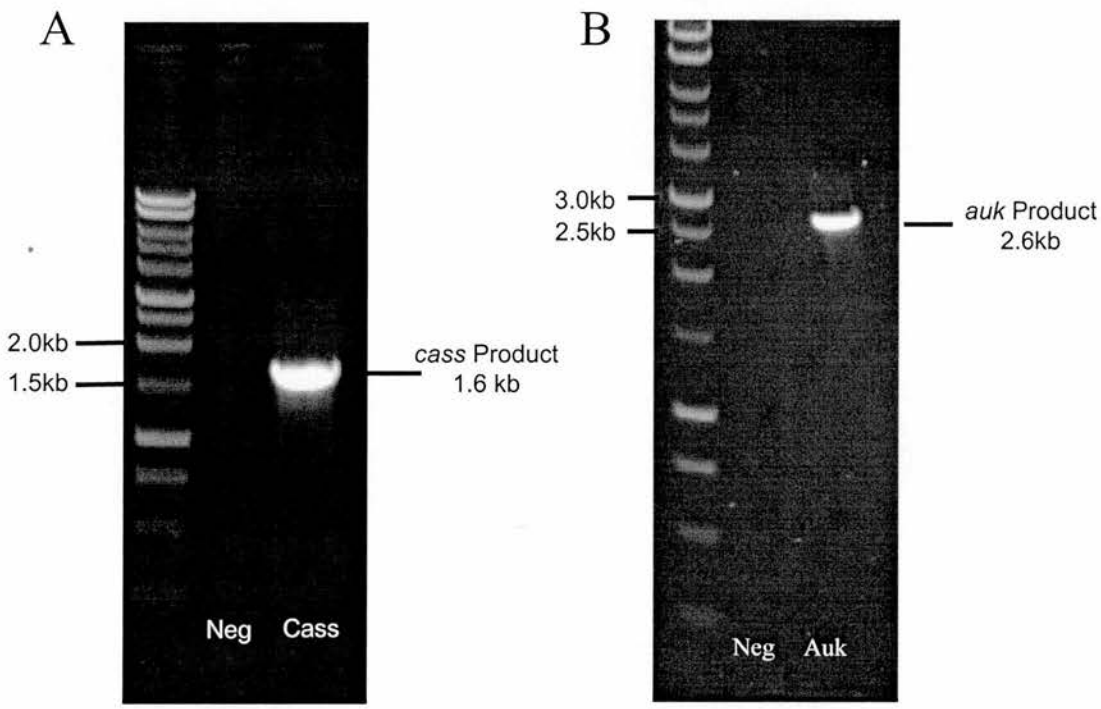


**Figure 6.1 High fidelity PCR of the *drosophila* genes, *cass* and *auk*.**

High fidelity PCR was performed on 25ng of template DNA for (A) *cass* and (B) *auk*. The negative control PCRs contained no template DNA. PCR products (5µl) were resolved on 1% agarose gels.

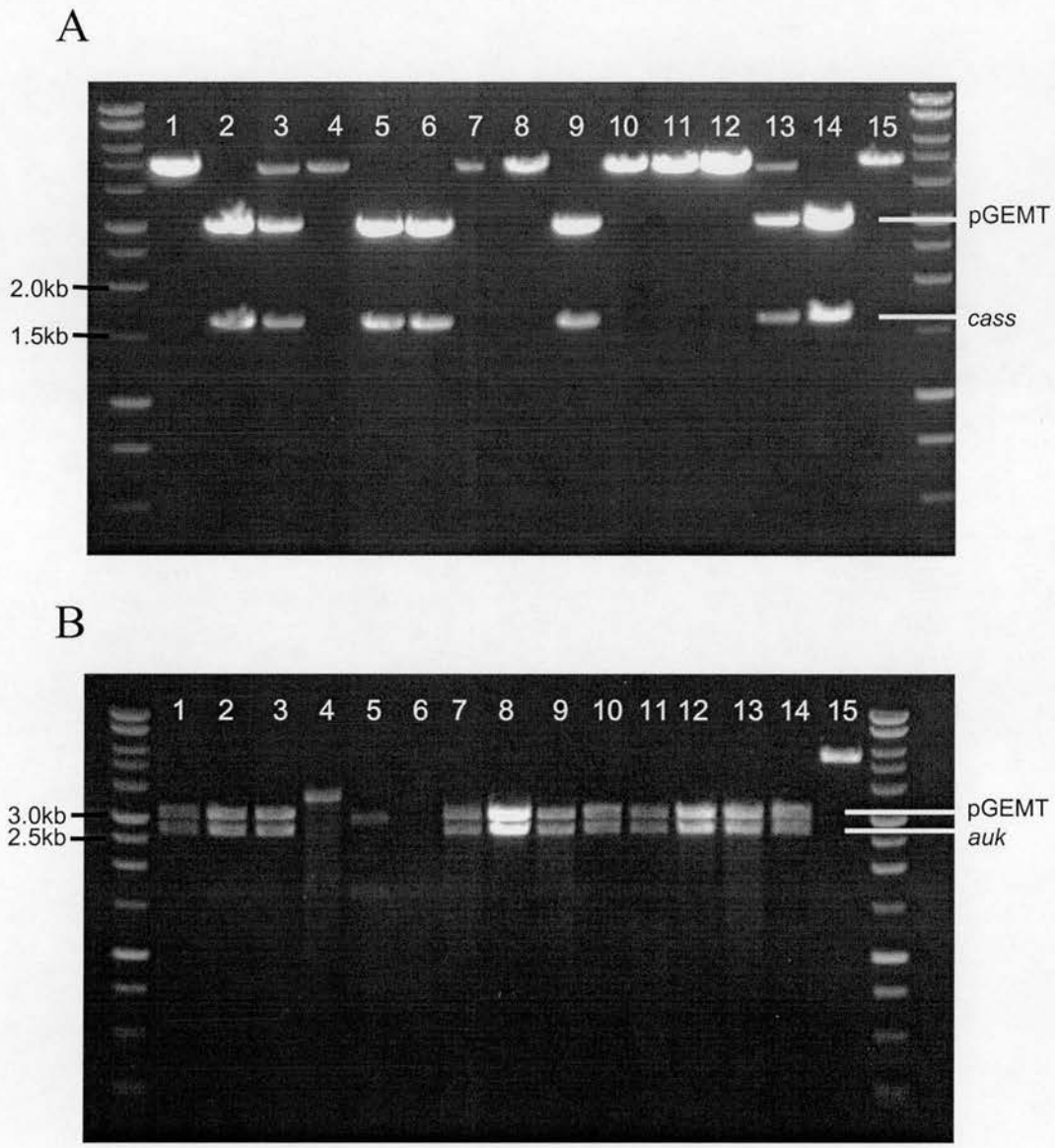


**Figure 6.2 Restriction digest of *cass* and *auk* ligations into the pGEMT T-vector.**

Mini-prepped DNA from (A) *cass* ligations (1-15) were digested with BamH1 and Hind III and (B) *auk* ligations (1-15) were digested with EcoRI and Hind III restriction enzymes and resolved on a 1% agarose gel against a 1kb DNA ladder. The white markers indicate the 3kb pGEMT vector and the cass/auk inserts.

Inserts from Cass-14 and Auk-13 clones were subsequently sub-cloned into the mammalian expression vector pCMV-Tag3B. Figure 6.3 shows restriction digests of amplified DNA from colonies from the ligation plates. The Cass ligation reaction only produced one colony, however, this was positive for the insert. This single Cass clone and Auk clone 4 were amplified and used in subsequent expression studies.

### 6.2.3 Expression of Cass and Auk in αβ-py cells

The pCMV-Tag3B expression vector contains a N-terminal myc epitope such that fusion proteins can be detected with the anti-myc (9E10) antibody. Cells transfected with Cass or Auk-3B expression vector were lysed and cell lysates were resolved by 10% SDS-PAGE. Following western blotting Figure 6.4 shows that the nitrocellulose membrane probed with the anti-myc antibody detected expression of both Cass and Auk proteins. No expression was observed from the Tag-3B control vector transfected cells. The myc-tagged Cass protein was expressed as a single 62kDa protein, while Auk was detected with an electrophoretic mobility of approximately 115kDa. Both myc-tagged proteins were also detected with a second anti-myc (A14) antibody. Utilising the Compute Mw tool on the ExPasy proteomics server at the Swiss Institute of Bio-informatics the predicted molecular weight of myc-tagged Cass and Auk was determined. Predicted molecular weights were 60kDa and 96kDa for myc-Cass and myc-Auk respectively.

### 6.2.4 Cellular localisation of Cass and Auk proteins

The presence of the myc-tag on the proteins allowed immuno-fluorescence to be performed on transfected cells. Cells were grown on glass coverslips and transfected with Cass-3B, Auk-3B or empty vector DNA. After 48h, the coverslips were stained with the anti-myc (9E10) antibody and visualised by fluorescence microscopy. Phase microscopy of cells transfected with Cass-3B or Auk-3B DNA showed cells with similar morphology to that observed with the empty vector control (Figure 6.5). Cells displayed the flat spread morphology observed with untransfected cells or cells transfected with the control vector.

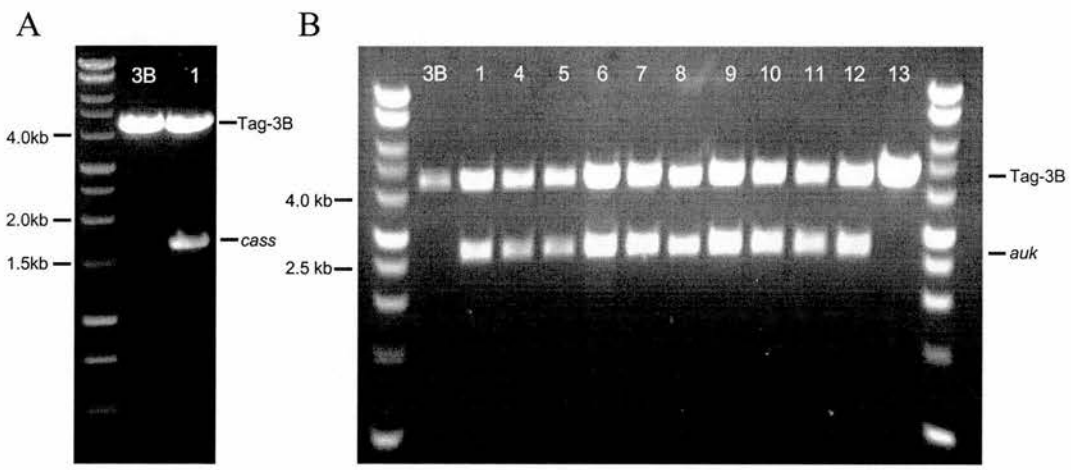


**Figure 6.3 Restriction digest of *cass/auk* ligations into the pCMV-Tag3B expression vector.**

Mini-prepped DNA from (A) *cass* ligations were digested with BamH1/Hind III and (B) *auk* ligations with EcoRI/Hind III restriction enzymes and resolved on a 1% agarose gel against a 1kb DNA ladder. The markers indicate the 4.3kb pCMV-Tag3B vector and the cass/auk inserts.

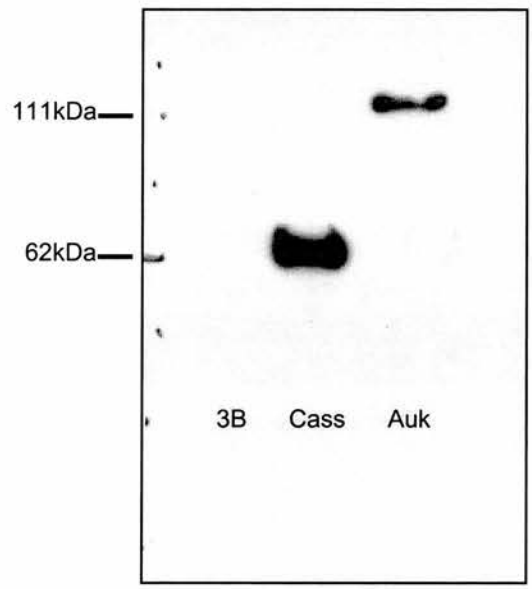


**Figure 6.4 Expression of Cass/Auk in αβ-py cells.**

Cells were transfected with either Cass-3B, Auk-3B expression vectors or 3B control vector (1μg). Western blots of cell lysates were probed with the anti-myc (9E10) antibody. The blots shown are representative of 3 experiments.

Fluorescence microscopy of empty vector transfected cells showed no staining with the anti-myc antibody. Cells transfected with Cass-3B showed a high level of Cass expression in a highly localised area. Merging both phase and fluorescence microscopy images of the cells showed that expression appeared to be localised within the nucleus of the transfected cells. Expression of the Cass protein was not observed outside the nucleus. In contrast, the Auk protein was expressed at a low level in αβ-py cells and expression was diffuse within the cell, furthermore the merged image did not show expression to be localised to a specific area of the cell.

## 6.3 Effect of Cass expression on integrin affinity

### 6.3.1 Cass does not suppress integrins

The Cass protein was shown to be expressed in αβ-py cells both by western analysis and by immuno-fluorescence. Figure 6.6A shows that expression of the Cass protein was optimal with transfection of 1μg of Cass-3B DNA, transfection with DNA quantity above 1μg resulted in lower expression which may be due to sub-optimal DNA/lipid ratios. The decrease in expression with increasing Cass-3B DNA was not due to DNA quantity, as all transfections were adjusted to 5μg of total DNA with the empty control vector.

Cells transfected with Cass-3B DNA were also assessed for integrin affinity modulation by flow cytometry. Figure 6.6B shows that transfection of increasing Cass-3B DNA into αβ-py cells did not cause any significant change ($P>0.05$) in integrin affinity compared to the empty 3B control vector. Cells transfected with Ras G12V DNA produced a characteristic loss in PAC1 antibody binding with an inhibition of $61.4 \pm 2.9\%$.

### 6.3.2 Cass cannot reverse Ras G12V-mediated integrin suppression

Transfection of Cass-3B DNA into αβ-py cells did not significantly affect integrin affinity. It was shown that R-Ras G38V is capable of reversing Ras G12V-mediated integrin suppression, however transfection of R-Ras G38V DNA alone does not

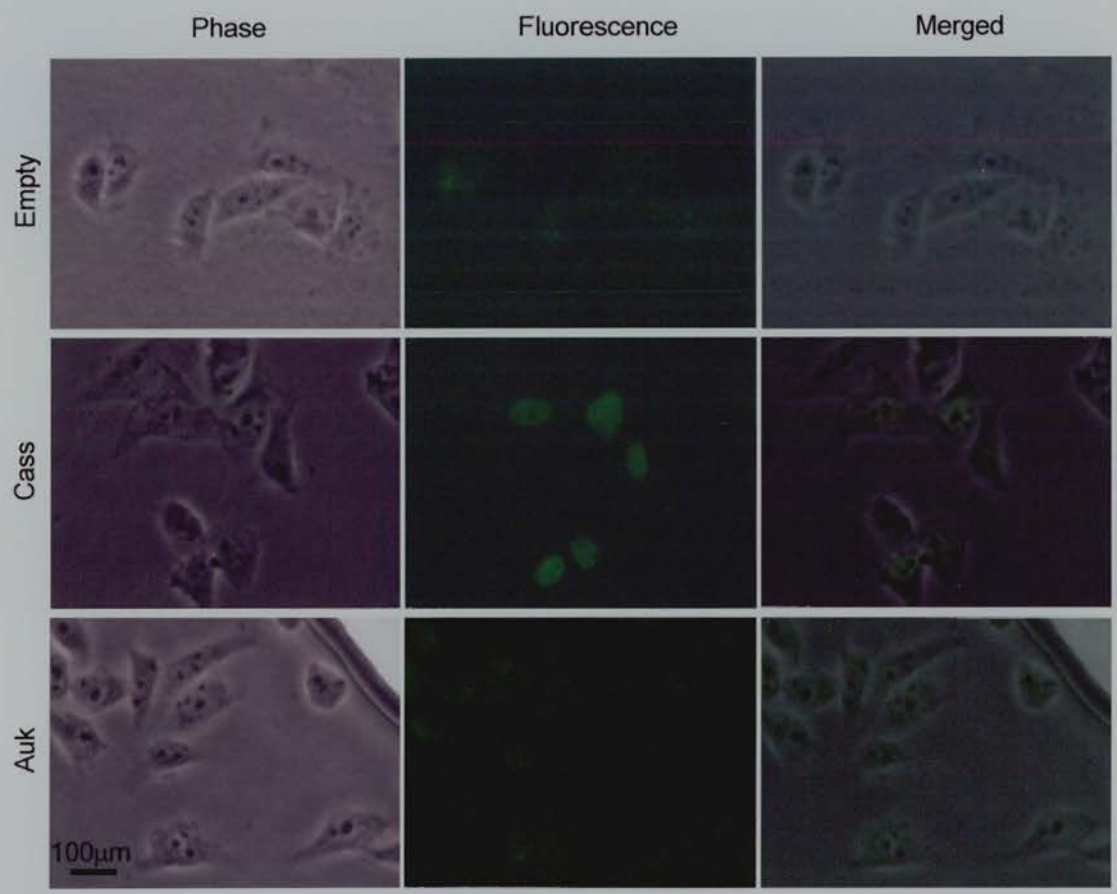


**Figure 6.5 Immunofluorescence of cass/auk expression.**

Cells transfected with Cass, Auk or empty vector (1μg) were permeabilised and stained with the anti-myc (9E10) antibody with an Alexa-green 488 secondary antibody. Panels show a phase contrast image (left) a fluorescence image (middle), and a merged image of the cells (right). Scale bar represents 100μm.

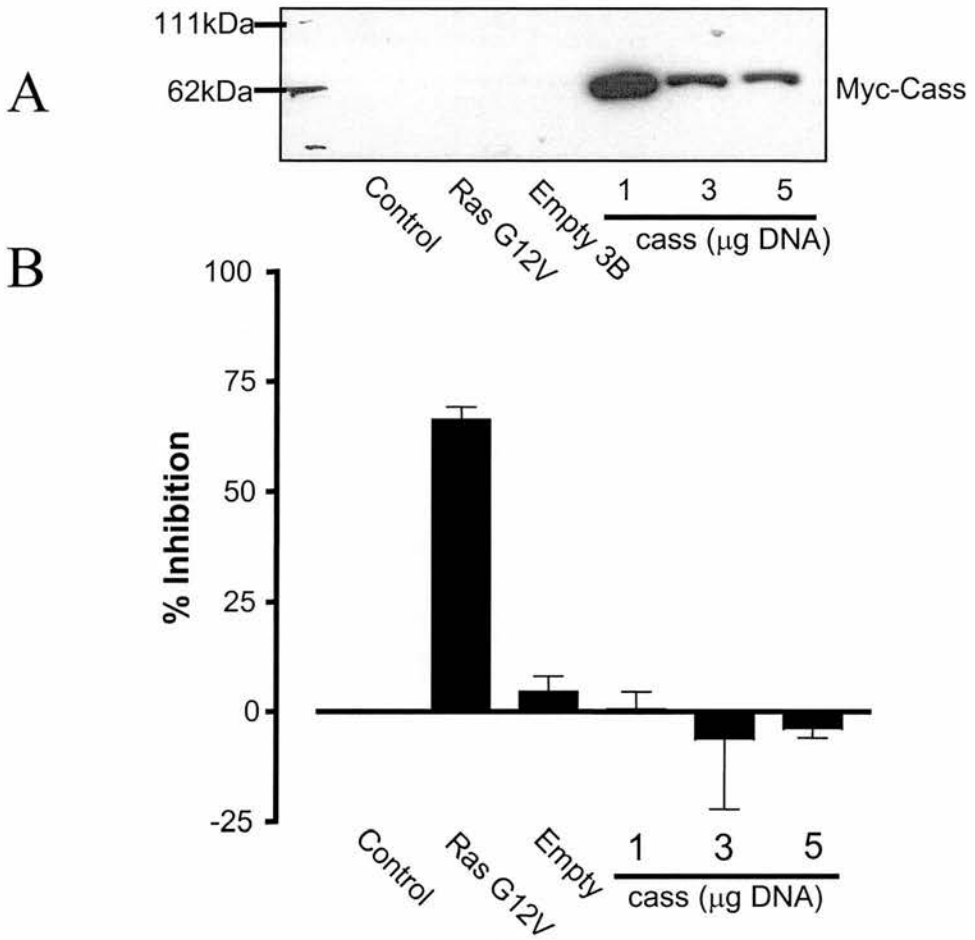


**Figure 6.6 Effect of Cass expression on integrin affinity.**

(A) Lysates from cells transfected with increasing cass DNA were probed with the anti-myc (9E10) antibody. Total DNA quantity was adjusted to 5μg in all transfections with the empty 3B control vector. The western blots shown are representative of 3 experiments. (B) Integrin affinity was determined in cells Ras G12V (1μg) was transfected as a positive control for suppression. Percentage inhibition was calculated in reference to cells transfected with control vector. The results shown are the mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA test.

significantly affect affinity modulation (Figure 4.11). We therefore sought to determine whether Cass-3B expression could affect integrin suppression by Ras G12V.

Cass-3B was co-transfected with Ras G12V DNA in αβ-py cells and expression of both proteins were analysed by western blots (Figure 6.8). Cass was expressed as a 62kDa protein at similar levels in both control and Ras G12V co-transfectants (Figure 6.7A). A decrease in Ras G12V expression was observed in co-transfections with either the control 3B vector or Cass-3B vector. In the presence of Cass-3B DNA, expression of Ras G12V was reduced compared to that in the presence of control 3B vector (Figure 6.7B).

Figure 6.7C shows that the reduction in Ras G12V expression in co-transfections with the control 3B vector did not affect integrin suppression (67.6 ± 1.3% and 66.9 ± 2.4% respectively). As with previous experiments Cass expression did not affect integrin affinity, however in co-transfections with Ras G12V a slight reduction in integrin suppression was observed (55.0 ± 3.3%), this difference was statistically significant ($P<0.05$), however, the decrease in Ras G12V expression in these cells may explain the fall in percentage inhibition.

## 6.4 Effect of Auk expression on integrin affinity

### 6.4.1 Auk expression does not affect integrin affinity

Similar to the experiments performed with Cass, Auk-3B was transfected into αβ-py and both expression and integrin affinity were determined. Figure 6.8A shows that the Auk protein was expressed in αβ-py cells. Expression was detected with the anti-myc (A14) antibody rather than the 9E10 clone. Results with the A14 antibody were more robust than with the 9E10 antibody. Transfection with 2μg of Auk-3B gave a marginal increase in expression to that observed with 1μg.

While Ras G12V suppressed integrins in transfected αβ-py cells (66.2 ± 1.3%), Auk did not affect integrin affinity compared to the empty 3B vector (Figure 6.8B).

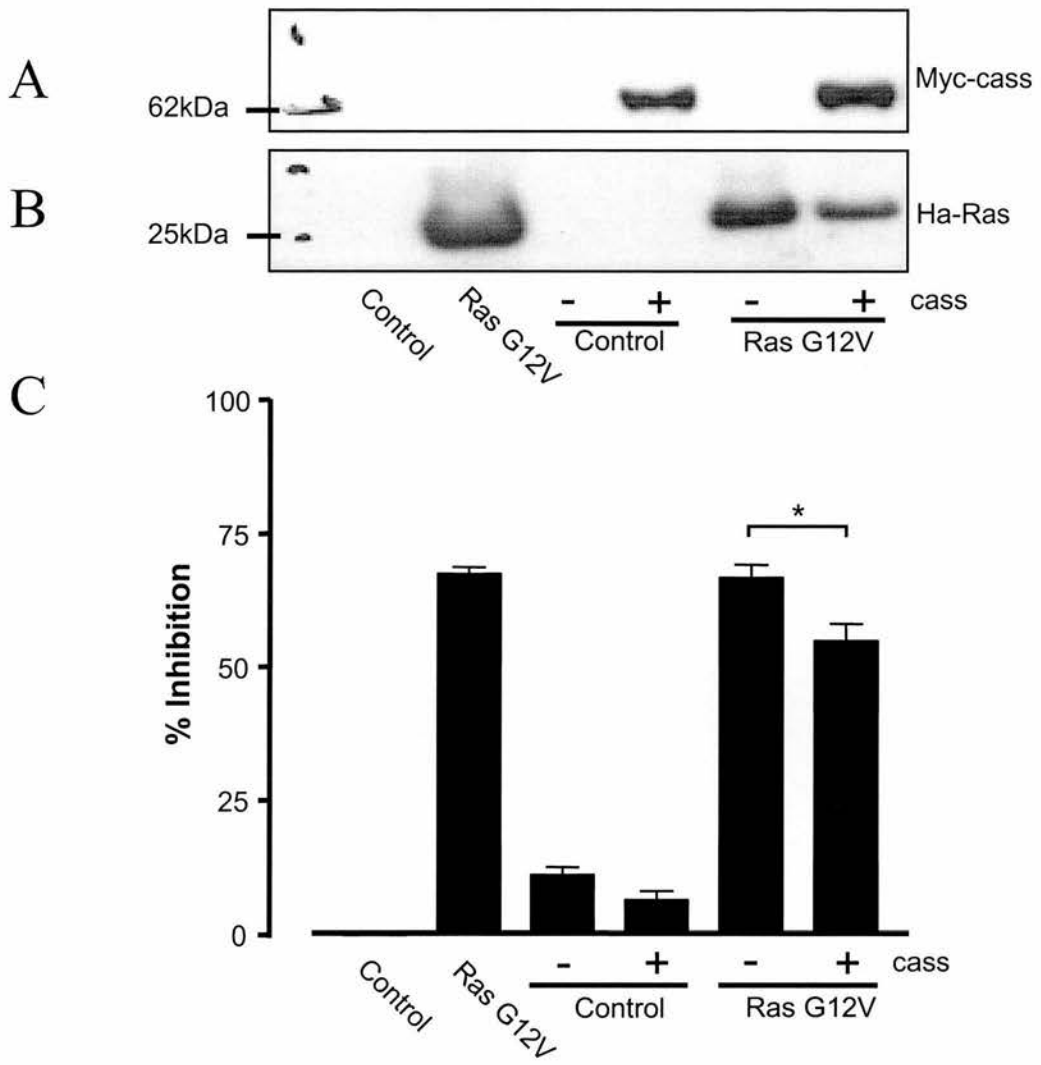


**Figure 6.7 Effect of co-expression of cass on Ras G12V mediated integrin suppression.**

Lysates from cells co-transfected with Ras G12V (1μg) and cass (1μg) or empty 3B vector (1μg) were probed with (A) anti-myc (9E10) antibody or (B) anti-Ha antibody. The blots shown are representative of 3 experiments. (C) Integrin affinity was determined in transfected cells. Percentage inhibition was calculated in reference to control vector. The results shown are the mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA test, asterisk indicates significant difference ($P<0.05$).

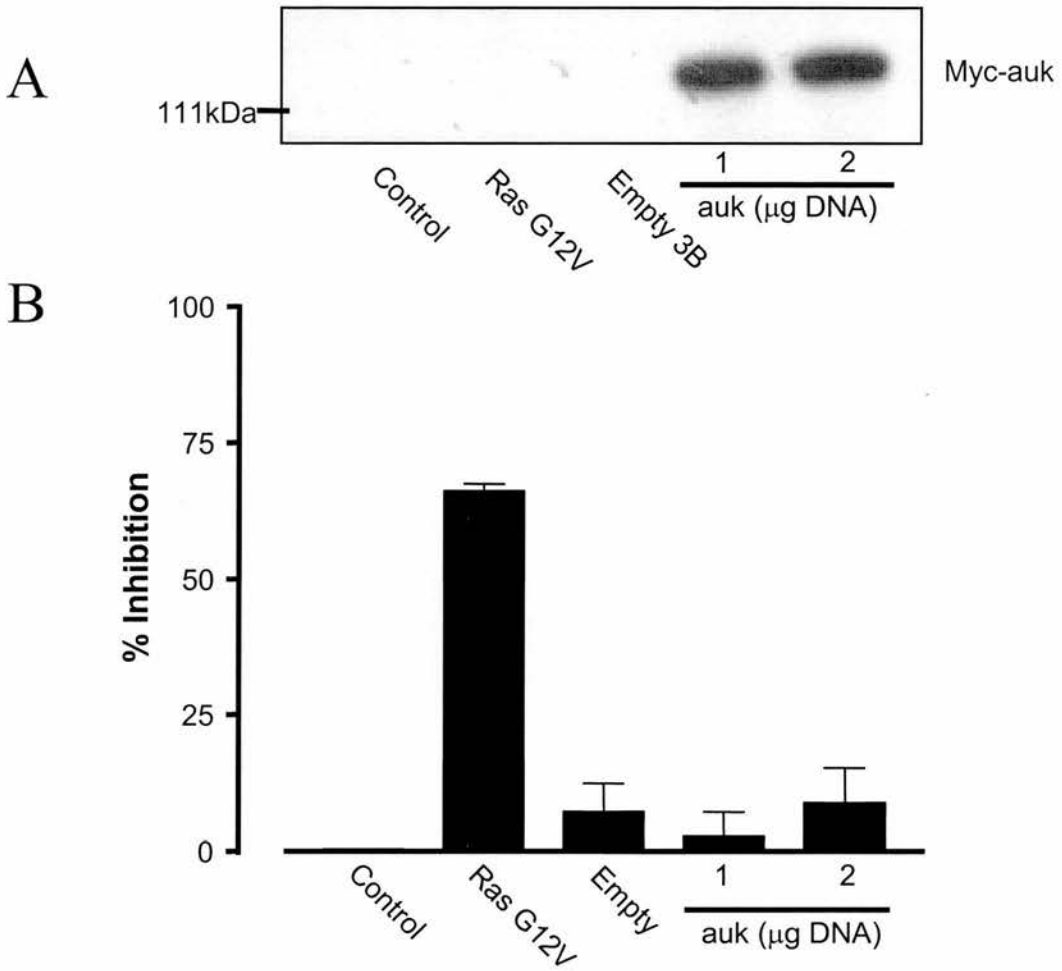


**Figure 6.8 Effect of auk expression on integrin affinity.**

(A) Lysates from cells transfected with increasing auk DNA were probed with the anti-myc (A14) antibody. Total DNA was adjusted to 2μg with the empty 3B control vector. The blots shown are representative of 3 experiments. (B) Integrin affinity was determined in cells transfected with increasing auk DNA. Ras G12V (1μg) was transfected as a positive control for suppression. Percentage inhibition was calculated in reference to control vector. The results shown are the mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA test.

Transfection of Auk (2μg) suppressed integrins by 8.8 ± 6.4% compared to 7.2 ± 5.2% for control 3B vector (2μg). Transfection of Auk DNA did not significantly increase ($P>0.05$) percentage inhibition.

### 6.4.2 Auk expression does not affect Ras G12V-mediated suppression

The effect of Auk expression on Ras G12V-mediated integrin suppression was determined in co-transfections between Auk and Ras. Figure 6.9A shows that Auk was expressed in co-transfections containing either control or Ras G12V DNA. Similar to the observations with Cass and Ras co-transfections, Ras G12V expression was reduced in the presence of control 3B DNA (Figure 6.9B). A further decrease in expression was not observed with Auk-3B co-transfections.

Co-transfection of either control 3B or Auk-3B with Ras G12V DNA did not affect integrin suppression mediated by Ras G12V. Figure 6.9C shows that the percentage inhibition in the presence of Ras G12V/control 3B was 59.4 ± 4.6 compared to 56.6 ± 5.6 when Auk-3B was co-transfected. The difference in percentage inhibition between Ras G12V transfected cells co-expressing Auk and control 3B vector were not significantly different ($P>0.05$). These results suggest that the Auk protein does not modulate the affinity of either the native chimeric integrin or the Ras G12V suppressed integrin.

## 6.5 Genetic screen of a SCLC cDNA expression library

In an attempt to identify novel components of the inside-out pathway involved in integrin affinity modulation a genetic strategy was adopted. Fenczik *et al.* (1997) have successfully used this strategy to identify CD98 from a CHO-K1 cDNA library that was able to reverse β1 tail integrin suppression. Ramos *et al.* (1998) showed that PEA-15 from a CHO-K1 cDNA library was able to reverse Ras G12V-mediated integrin suppression. A small cell lung carcinoma (SCLC) cDNA expression library

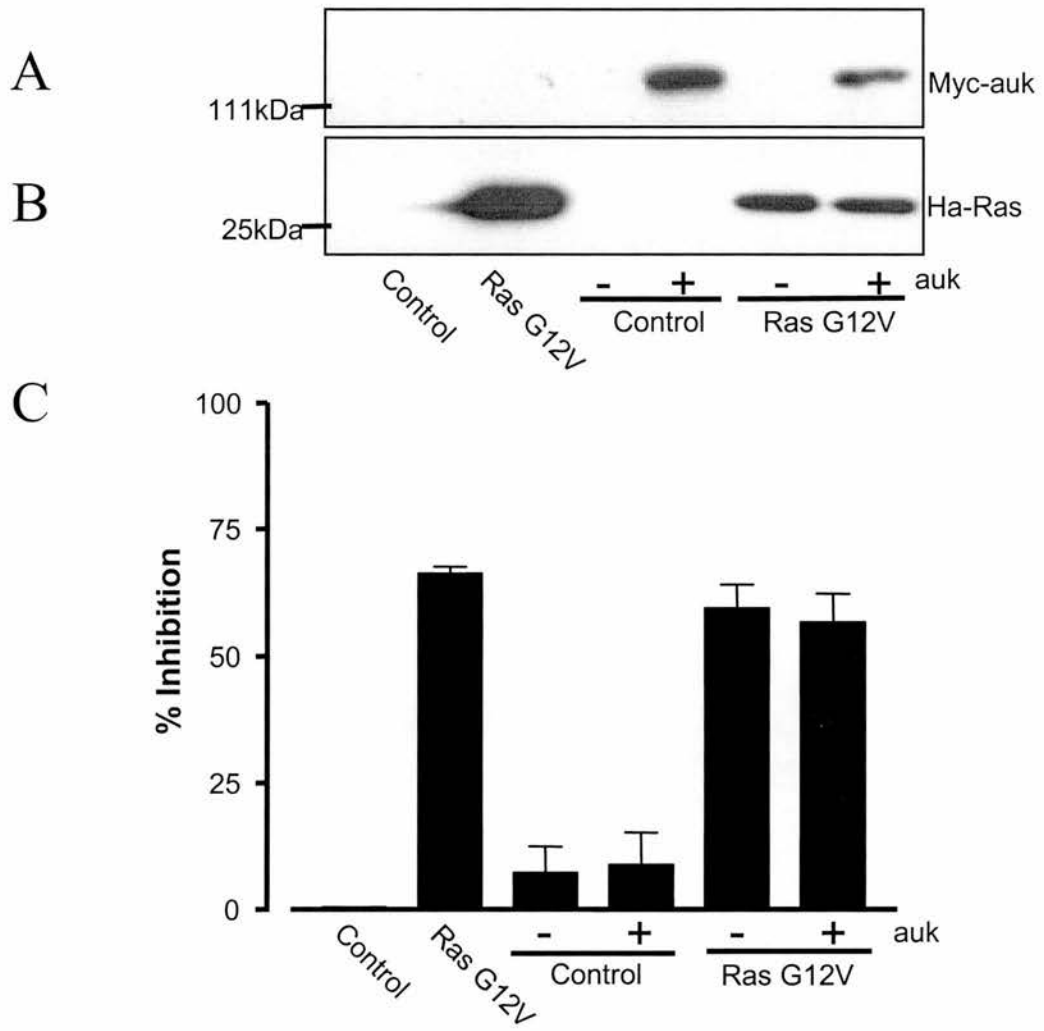


**Figure 6.9 Effect of co-expression of auk on Ras G12V mediated integrin suppression.**

Lysates from cells co-transfected with Ras G12V (1μg) and Auk (2μg) or empty vector (2μg) were probed with (A) anti-myc (A14) antibody or (B) anti-Ha antibody. The western blots shown are representative of 3 experiments. (C) Integrin affinity was determined in co-transfected cells. Percentage inhibition was calculated in reference to control vector. The results shown are the mean ± SEM of three independent experiments. Statistical analysis was performed by one-way ANOVA test.

(H33) was obtained from the MRC Human Genome Mapping Project and used to screen for genes capable of integrin suppression.

The library was amplified in commercially competent TOP10/P3 E.Coli (Invitrogen) in order to maximise the number of clones recovered. The cDNA library cloned in a pCDM8 expression vector contains a CMV promoter and polyoma T antigen origin of replication to facilitate autosomal replication in the αβ-py cells. Figure 6.10 shows an XhoI restriction digest of the SCLC library and pCDM8 empty vector. The XhoI restriction site flanks the insert cloning site and digestion of the pCDM8 vector produced a band of 4.0kb. Digests of the SCLC library showed the pCDM8 backbone at 4.0kb together with two major bands at 2.5kb and 12.0kb. A DNA smear was also observed stretching from this 12.0kb band to approximately 500bp. A similar restriction pattern was also observed with digestion of a macrophage cDNA library with the XhoI enzyme.

### 6.5.1 Screening of the SCLC library transfected cells

Preliminary experiments were performed to titrate both antibody dilutions and DNA quantities for the large number of cells used during the cell sort. Topro3 used for cell viability staining during affinity determination experiments was replaced with the 7-AAD viability stain due to the absence of an FL-4 channel on the cell sorter. Test sorts were performed with Ras G12V a known suppressor of integrin affinity to optimise both the cell sorting instrument settings (PMT voltages, compensation and sort gates) and to validate the HIRT extraction protocol to rescue transfected plasmids. From these tests sorts we were able to obtain colonies from the sort gate that were transfected with Ras G12V DNA.

Cells transfected with the SCLC cDNA library were sorted according to the previous test experiments. Using Ras G12V transfected cells as a positive control, the sort gate contained a large number of cells with reduced PAC1 binding compared to the pCDM8 control vector (Figure 6.11). Cells transfected with the library displayed a similar profile as that observed with the pCDM8 control vector. As a result of the large number of cells used in the sorting process, only a fraction of the analysed cells

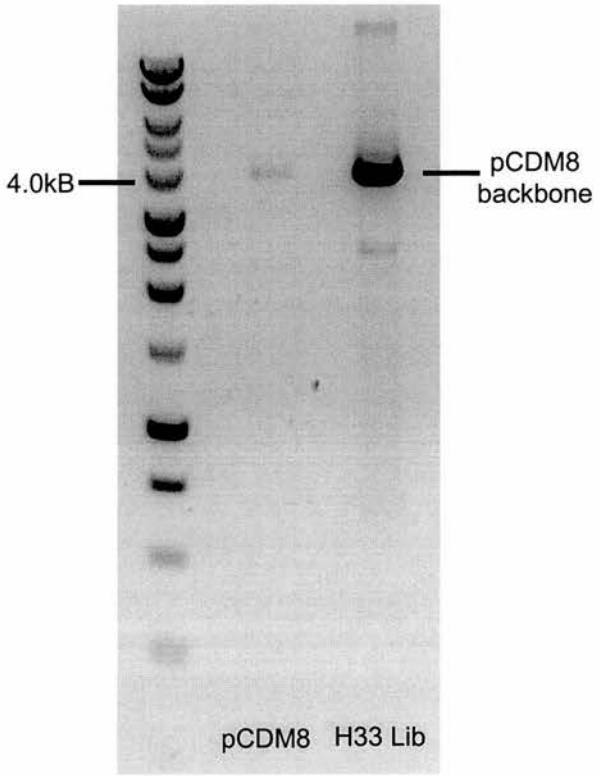


**Figure 6.10 Digest of H33 SCLC cDNA expression library.**

H33 SCLC expression library DNA (1μg) was digested with 10 units XhoI restriction enzyme; the empty pCDM8 vector (50ng) was also digested. Digests were resolved on a 1% agarose gel containing ethidium bromide.

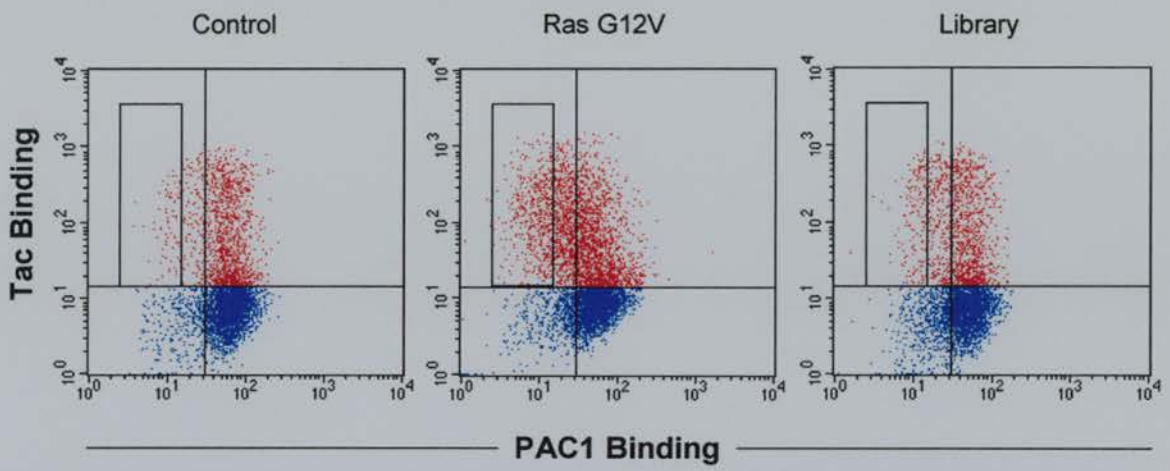


**Figure 6.11 Expression of H33 SCLC cDNA library in αβ-py cells.**

Cells were transfected with pCDM8 control vector (4μg), Ras G12V (2μg) or H33 SCLC library (4μg). After 48 hours, cells were stained for Tac expression and PAC1 binding. Tac positive cells are shown in red and the sort gate in the upper left quadrant represents cells that were collected by the cell sorter.

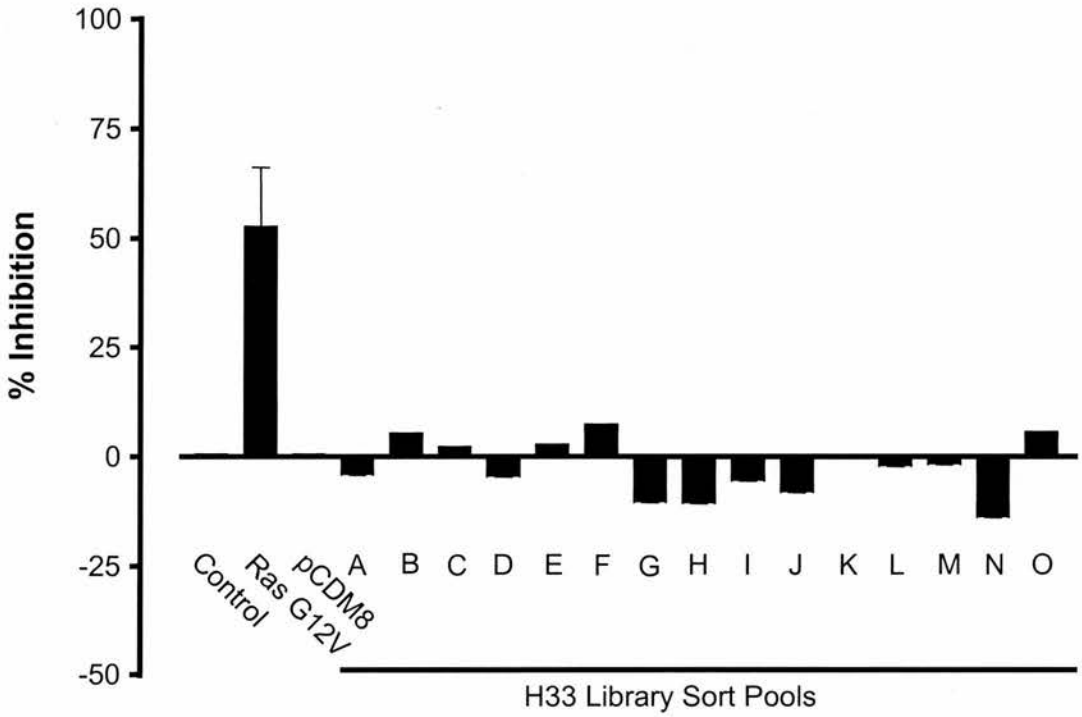


**Figure 6.12 Screen of library pools for effects on integrin affinity.**

Integrin affinity was determined in cells transfected with DNA (4μg) isolated from library pools. Each pool contained transformed bacterial cultures from 10 separate colonies from the initial H33 library sort. The results represent data accumulated from 6 separate experiments with percentage inhibition calculated in reference to the pCDM8 empty vector.

are shown in this dot blot. Hirt extraction was performed on the cells collected from the sort to extract the transfected plasmids within these cells. Plasmid DNA was digested with the DpnI restriction enzyme prior to transformation of TOP10/P3 E.Coli.

One hundred and fifty three colonies were obtained during this transformation. These colonies were cultured and used to generate screening pools. Individual cultures were pooled into groups of 10 and amplified to enable plasmid purification. DNA pools A-O were transfected into αβ-py cells and their effect on integrin affinity was determined (Figure 6.12). In reference to the pCDM8 control vector, DNA pools A-O did not significantly alter the integrin affinity of the chimeric integrin. Cells were responsive to suppressive signals as Ras G12V was able to reduce PAC1 binding in all the screening experiments.

The expression cloning strategy has previously been successful in identifying novel components of integrin affinity modulation. In our hands, the screen failed to identify genes from the SCLC library that may be involved in integrin suppression.

## 6.6 Discussion

In this chapter we have cloned and expressed two *drosophila* genes identified from a genetic screen in αβ-py cells. Proteins, cassowary and auk failed to modulate the affinity of the native chimeric integrin and were not capable of reversing Ras G12V-mediated integrin affinity. Screening of a SCLC cDNA expression library also failed to identify genes involved in integrin suppression.

Integrins and integrin-mediated adhesion have been shown to be crucial for *drosophila* development (Brown, 2000; Gotwals *et al.*, 1994). Genetic screens in drosophila have identified several novel mutants that display the blistered wing phenotypes associated with a loss of integrin-mediated adhesion (Prout *et al.*, 1997; Walsh and Brown, 1998). Several of these mutants have been identified and cloned including cass, auk and kakapo. Kakapo encodes a cytoskeletal adapter protein related to plectin or dystrophin. The protein appears to be required to maintain epidermal adhesion to the muscle through interactions with integrins and the actin and microtubule cytoskeleton (Gregory and Brown, 1998). Unlike kakapo, sequence analysis of cass and auk failed to identify any significant regions of interest towards integrin-mediated adhesion.

Cass and auk were both expressed in αβ-py cells as 62 and 115kDa proteins. The size of the cass protein was comparable to the predicted mass of myc-tagged cass (60kDa). A 20kDa discrepancy was observed between the predicted weight of auk (96kDa) and that observed in transfected αβ-py cells. The size discrepancy may reflect post-translational modifications, approximately seven O-GlcNac glycosylation sites were predicted to exist on the auk protein.

Expression of Cass or Auk failed to effect integrin affinity. The localisation of Cass to the nucleus may explain the inability of Cass to interact with the inside-out signalling pathway. The same explanation cannot be used for Auk as it appears to be expressed diffusely throughout the cell.

Sequence analysis of Cass revealed that orthologues exist in several species including humans called anti-apoptosis clone 11 (AAC-11). A truncated form of AAC-11 (1kb) was initially identified by its ability to protect fibroblast from growth factor withdrawal induced apoptosis, full length AAC-11 did not protect against apoptosis (Tewari *et al.*, 1997). AAC-11 contains a C-terminal leucine zipper motif but lacks a DNA-binding domain that appears to be required for the anti-apoptotic effects. The leucine zipper motif is not well conserved in the Cass sequence. The role of Cass in drosophila cell apoptosis is currently being investigated by Dr. N. Brown (Cambridge). AAC-11 has also been shown to be up-regulated in metastatic cervical tissue compared to normal tissue. Over-expression of AAC-11 in cervical cell lines altered matrix metalloproteinase (MMP) expression, increased soft agar colony formation, invasion into matrigel and increased cell adhesion to laminin (Kim *et al.*, 2000). The ability of AAC-11 to alter MMP expression may reflect a role of AAC-11 in transcription regulation. The localisation of Cass to the cell nucleus in αβ-py cells may explain the ability of AAC-11 to regulate gene expression. As the authors did not examine integrin levels in transfected cells, AAC-11 may also alter integrin expression levels. The chimeric integrin contains the cytoplasmic domain of the $\alpha_{6A}\beta_1$ laminin receptor. Cass did not affect the affinity of this integrin, therefore the increase in adhesion to laminin may be through changes in integrin expression levels, avidity changes or through other laminin receptors. AAC-11 has recently been shown to be a marker of increased tumour aggressiveness in non-small cell lung cancer patients (Sasaki *et al.*, 2001). Cass/AAC-11 does not appear to have a direct role in modulating integrin affinity, however its role in apoptosis and tumour biology merits further study.

Auk has shown sequence similarity to proteins of the La family, a family of possible RNA binding proteins. The RNA binding domain within these proteins is not conserved in Auk indicating that Auk may not play a role in RNA binding. Neither Cass or Auk genes have any recognisable protein kinase domains, protein interaction domains (SH2/SH3) or lipid binding regions, their direct role in cell signalling is therefore debatable. Their role in integrin-mediated adhesion may therefore rely on

protein-protein interactions with other proteins. Several of the mutants identified in the initial screen remain to be cloned; it will be interesting to determine whether these proteins have any role in integrin affinity modulation.

An alternative approach to identify genes involved in affinity modulation was to perform a genetic screen of a SCLC expression library. Unfortunately the screen failed to identify any genes that could mediate integrin suppression. Only 150 clones were obtained from the E.coli transformation by the Hirt plasmid extract. This was a small number considering that 50,000 events were collected within the sort gate. While event number does not exactly correlate with cell number, it does suggest that a significant proportion of cells collected may not have contained any library plasmid. The DpnI digestion step also drastically reduces the number of colonies obtained. The DpnI restriction site requires DNA methylation; plasmids that have replicated in the αβ-py cells lose this methylation and are therefore resistant to digestion. This step removes plasmids that fail to replicate in αβ-py cells including the Tac-$\alpha_5$ reporter construct and should reduce background noise within the screen. If a large proportion of the collected cells contain plasmids that have not replicated this may explain the small number of colonies recovered. To address these problems, we can increase the sort gate to increase the number of cells collected or use other expression libraries. A second screen is currently underway in the laboratory; a PMA treated macrophage library is being screened for genes that can reverse Ras G12V suppression. Increasing the sort gates, a greater number of colonies were obtained; the colonies have been pooled and amplified. Screening of the pools by flow cytometry is currently underway, positive pools have been identified and are under further study (R. Buttery, University of Edinburgh personal communication).

In summary we have cloned and expressed two *drosophila* genes into αβ-py cells, unfortunately these genes had no affect on integrin affinity. We have also performed a genetic screen of a SCLC cDNA expression library. Screening of this library, did not identify any genes that could suppress integrins.

# DISCUSSION: CHAPTER 7

## Concluding Remarks and Future Work

Integrin affinity modulation is essential to the function of integrins. A conformational change arises through intracellular signals acting upon the cytoplasmic tails of integrins (Ginsberg *et al.*, 1992). H-Ras (Ras G12V) can suppress a constitutively active integrin in CHO cells (Hughes *et al.*, 1997). The aim of this thesis was to elucidate the mechanism of Ras G12V-mediated integrin suppression.

Ras effector mutants revealed that suppression occurred through Raf-dependent, (Ras (G12V, T35S)) and Raf-independent, (Ras (G12V, E37G)) pathways. Integrin suppression by Ras (G12V, Y40C) is currently under further study. Figure 7.1 shows the proposed mechanism of integrin suppression downstream of Ras signalling. Activation of RalGEFs by Ras (G12V, E37G) was not responsible for suppression; however, activation of Raf by Ras (G12V, T35S) did facilitate integrin suppression. Stimulation of ERK1/2 activity by Raf signalling was not required for suppression, as a constitutively active ERK2 failed to suppress integrins, while the Raf-BxB T481A mutant mediated suppression in the absence of MEK activation. Sensitivity of Raf-mediated integrin suppression to MKP-1 and MKP-3 expression implicated an ERK-like protein with a TEY consensus sequence.

Both H-Ras and R-Ras can modulate integrin affinity. With identical effector domains and a high degree of sequence identity adjacent to the effector domain suggests that both H-Ras and R-Ras may share common effectors, although R-Ras specific effectors that have yet to be identified may mediate unique R-Ras functions. H-Ras has recently been localised to lipid-raft microdomains within the plasma membrane (Prior *et al.*, 2001), microdomain localisation of R-Ras has not been reported. The hypervariable sequence of H-Ras was essential for targeting to the lipid-rafts, K-Ras4B, which contains a polybasic lysine motif, was excluded from

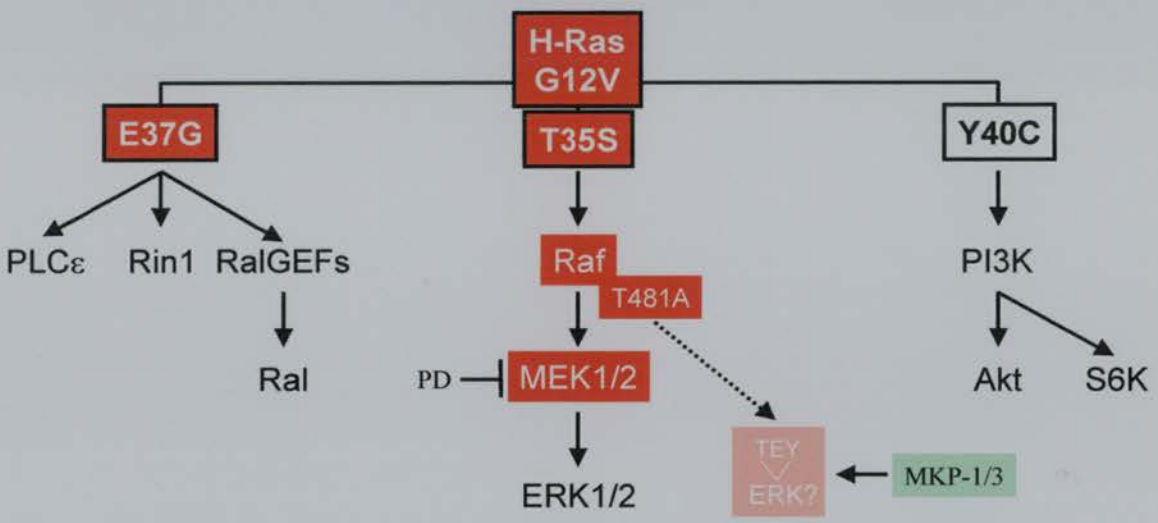


**Figure 7.1 Proposed pathway of Ras G12V-mediated integrin suppression.**

Ras G12V and Ras effectors mutants are shown in the black lined boxes. Red boxes denote proteins that can mediate integrin suppression. The pink box indicates the proposed ERK-like protein. For clarity, Ras effectors AF6, PKCζ and MEKK1 have not been shown. PD – PD098059 (MEK1 inhibitor).

lipid-rafts and localised to the bulk plasma membrane. The differential microdomain localisation of the Ras isoforms may contribute to the varying ability of the Ras isoforms to activate Raf-1 and PI3-kinase (Voice *et al.*, 1999; Yan *et al.*, 1998). It will be interesting to determine whether K-Ras4B is as potent as H-Ras in modulating integrin affinity.

Substitution of the H-Ras hypervariable sequence with that of R-Ras drastically reduced the transforming ability of this chimeric protein (Lowe *et al.*, 1988). H-Ras and R-Ras chimeras have also shown that sequences within the C-terminus are important for specifying the unique effects on integrin affinity by H-Ras and R-Ras (personal communications P. Hughes, Scripps Research Institute, USA and J. Love, University of Edinburgh, UK). The chimeras have also revealed that H-Ras can mediate integrin suppression in the absence of ERK1/2 activation in correlation with data in this thesis. Membrane localisation of these chimeras may provide clues to explain the unique properties of H-Ras and R-Ras towards integrin affinity.

Hughes *et al.* (1997) have shown that activation of endogenous Ras with active Src can mediate integrin suppression. Ras activation by growth factors has yet to be described to modulate integrin affinity. One can speculate that integrin suppression is a regulated cellular process and that Ras activition e.g. during cell cycle progression, may not always correlate with integrin suppression. Whether spatial and temporal Ras activation regulates the ability of Ras to mediate integrin suppression remains to be determined.

Integrin suppression by the Raf-independent, Ras (G12V, E37G) pathway was not blocked by a dominant negative Ral construct although Ral activity was completely inhibited. Ras effectors phospholipase Cε and Rin1 bind to Ras G12V and of the three effector mutants, preferentially bind to Ras (G12V, E37G) (Han *et al.*, 1997; Kelley *et al.*, 2001). Future work with Ras (G12V, E37G) must therefore take into account these effector pathways in addition to RalGEFs.

The relevance of PLCε activation on integrin affinity could be determined both pharmacologically and by gene expression studies. A PLC inhibitor, U-73122 has

been shown to inhibit Ras G12V stimulated adhesion of a haematopoietic cell line to fibronectin (Shibayama *et al.*, 1999), whether this inhibitor is active towards PLCε has not been determined. The Ras effector mutant Ras (Q61L, D38N) can differentiate between activation of RalGEFs and PLCε (Kelley *et al.*, 2001), a similar mutation in the Ras G12V background would enable the significance of PLCε activation to be determined, alternatively PLCε mutants that fail to interact with Ras could be used. PLCε also contains a cdc25 homology domain (GEF activity) that can activate Rap1; deletion of this region prevents Rap1 activation (Jin *et al.*, 2001) and could be used to dissect a possible role of PLCε towards integrin affinity.

The biological role of Rin1 is currently unclear, isolated Ras binding domains (RBD) of Rin1 can inhibit Ras induced transcription, though this was not reproduced with full length Rin1 (Han *et al.*, 1997). Rin1Δ, which lacks the RBD, would enable activation of Rin1 to be studied further.

Raf-dependent integrin suppression is independent of MEK and ERK1/2 activation. The ability of MEK to mediate integrin suppression is a result of a feedback activation of Raf (Zimmermann *et al.*, 1997); MEK1-DD (active construct) can increase Raf activity in αβ-py cells (J. Love personal communication). A growing body of evidence supports the existence of MEK independent pathways downstream of Raf and alternative Raf effectors (Chapter5). The ability of MKP-1 (Chapter5) and MKP-3 (preliminary results) to reverse Raf-mediated suppression indicates that a TEY containing ERK protein may mediate suppression. Possible candidates include ERK5 (English *et al.*, 1999) and p97 ERK5-related protein (Janulis *et al.*, 2001), cloning of the latter is of particular interest as the characteristics of this protein closely fit with the current data of Raf-mediated integrin suppression. Screening of novel Raf phosphorylation targets may also provide an insight into the mechanism of integrin suppression (W. Kolch, personal communication). An in-gel kinase assay may enable the detection of the novel ERK activity stimulated by Raf-BxB T481A.

This thesis has concentrated on the mechanism underlying Ras G12V-mediated integrin suppression. The cellular consequence of such integrin suppression, however

remains to be answered. Ras expression can modulate integrin-dependent cellular functions including fibronectin matrix assembly (Brenner *et al.*, 2000), adhesion (Shibayama *et al.*, 1999) and migration (Voice *et al.*, 1999). Cell morphology, altered upon Ras effector mutant expression may partially correlate with integrin suppression although cytoskeletal changes are also required (Chapter4). Fibronectin matrix assembly was inhibited by Ras transformation (Brenner *et al.*, 2000), whether the Ras effector mutants E37G and T35S have a similar effect will be interesting to determine. Voice *et al.* (1999) has shown that the Ras isoforms differ in their ability to stimulate cell mobility (Voice *et al.*, 1999) possibly due to differential stimulation of Ras effector pathways; changes in effector pathway utilisation by the Ras effector mutants may also modulate cell mobility. Mobility changes of transfected cells could be assessed by both migration in Boyden chambers or via time-lapse microscopy (LaFlamme *et al.*, 1994). Cell migration across a matrix requires the transient adhesion and de-adhesion of the cell at the leading and trailing edge respectively (Lauffenburger and Horwitz, 1996). These changes are in part mediated by the cycling of focal adhesions during cell migration (Petit and Thiery, 2000). Spatial changes in integrin affinity between the leading edge and the trailing edge could be assessed by confocal microscopy with the PAC1 antibody. Integrin suppression at the trailing edge could allow retraction of the cell and similarily integrin activation at the leading edge may provide the traction required for migration. Redistribution of integrin affinity modulators may contribute to the spatial changes in integrin affinity, confocal microscopy would show whether Ras proteins concentrate to the trailing edge of a cell during migration.

Ras G12V stimulates adhesion of haematopoietic cells to fibronectin through activation of $\beta_1$ integrins; this contradicts the ability of Ras G12V to suppress integrins in $\alpha\beta$-py cells. The inability of Ras G12V to activate resting $\alpha_{IIb}\beta_3$ in CHO cells (Sethi *et al.*, 1999), suggests that affinity modulation by Ras expression may dependent on cell type and on the integrin affinity state. Adhesion of Ras G12V transfected $\alpha\beta$-py cells on fibrinogen (by chimeric integrin) would determine whether Ras-mediated integrin suppression affects cell adhesion.

This current study has furthered our understanding of Ras-mediated integrin suppression; future work may allow the identification of potential targets for therapeutic intervention. Such developments may allow the regulation of integrin-dependent processes. Platelet aggregation could be modulated to prevent excessive and damaging arterial obstructions, leucocyte infiltration could be restricted during chronic inflammatory diseases and possibly reduce tumour metastasis by inhibiting tumour cell detachment at the primary tumour mass.

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